The role of elongation factor EF-P in translation and in copy number control of the transcriptional regulator CadC in *Escherichia coli*

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Abbreviations

σ^S	stationary phase specific sigma factor (*rpoS*)
AAA^+	ATPases associated with various cellular activities
aa	amino acid
amp^R	ampicillin resistance
AR	acid resistance
ara	arabinose
A-site	aminoacyl-site
ASP	acid shock protein
ATP	adenosine-5´-triphosphate
bp	base pair
cAMP	cyclic adenosine-5´-monophosphate
cat	chloramphenicol acetyltransferase
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EF	elongation factor
E-site	exiting-site
Fig.	Figure
gm^R	gentamycin resistance
h	hour (s)
GTP	guanosine-5´-triphosphate
His_6-tag	affinity tag containing 6 histidine residues
HTH	helix turn helix
IF	initiation factor
kDa	kilodalton
LB	Lysogeny Broth
mRNA	messenger ribonucleic acid
MU	miller units
min	minute (s)
N-formyl-methionyl-$tRNA_i$	fMet-$tRNA_i$
Ni^{2+}-NTA	nickel-charged resin

npt	neomycine phosphotransferase
ODx	optical density at the wavelength x nm
OL	overlap
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDB	Protein Data Bank (www.pdb.org)
pmf	proton motive force
ppGpp	guanosine tetraphosphate
P-site	peptidyl site
PTC	peptidyltransferase center
P_x	promoter of gene *x*
qRT-PCR	quantitative real time PCR
rbs	ribosomal binding site
RF	release factor
SDS	sodium dodecyl sulfate
sec	second (s)
tet^R	tetracycline resistance
TM	transmembrane domain
tRNA	transfer ribonucleic acid
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

Nomenclature

Gene products are numbered in a way that the first methionine of the wildtype protein is designated “1” in the amino acid sequence. N-terminal affinity tags or linker sequences are left unconsidered.

Positions of nucleotides indicate the distance from the translational start site (A of ATG is +1).

Gene deletions are depicted by the symbol Δ, gene replacements as follows: replaced gene::new gene. When only parts of the gene were deleted, the missing nucleotides are specified (for example: $yjeK_{643\text{-}1029}$).

Amino acids are designated in one-letter or three-letter code. For single amino acid substitutions, the native amino acid is named in front of the corresponding amino acid position in the protein. The substituted amino acid is terminally added (for example: CadC-P122A). The exchange of the CadC proline cluster against alanines and a serine is designated as P/A or PPPIP/AAAIS.

Cells containing wildtype *efp* are termed efp^+ cells. *efp* deletion mutants are designated as efp^- cells.

Fusion proteins consisting of truncated CadC and full-length LacZ are designated as CadC´-LacZ.

Contributions

Figure 11 and Figure 13:

The design for the experiments was done by Susanne Ude (SU), Dr. Jürgen Lassak (JL), Prof. Dr. Kirsten Jung (KJ). qRT-PCR experiments were done by JL to verify and complete Northern Blot results obtained by SU. Experiment for Fig. 13B was performed by SU and JL.

Table 5, Figure 14, 16, 18, 19, 20, 22, 27C:

The experiments were designed by SU, JL and KJ. The practical work was done by SU (Fig. 14, Fig. 16C, Fig. 18, Fig. 22), JL (Fig. 16B, Fig. 27C, Fig. 19, Fig. 20) and Christina Krönauer (Table 5).

Figure 21 and 25B:

The experiments were planned by SU, JL, KJ, Dr. Daniel Wilson and Dr. Agata Starosta. The practical work was done by JL (construction of pET16b-CadC templates) and Dr. Agata Starosta (in vitro translation assay).

Figure 23 and Figure 24:

Dotblots were performed by JL.

Fig. 24C: The experiment was planned by SU, qRT-PCRexperiment was performed by JL.

Table A1:

Table A1 was created by Dr. Daniel Wilson.

Experiments which are not mentioned here were made by SU.

Portions of figures, tables and text were previously published in a different form in:

Ude S., Lassak J., Starosta A.L., Kraxenberger T., Wilson D.N., Jung K. (2013): Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. *Science* **339**:82-85.

1. Introduction

1.1. Acid stress adaptation

The interplay between humans and microbes for human health is highly important and was first described over 100 years ago by Pasteur (Schottelius, 1902). The human surfaces and the gastrointestinal tract (GI) are inhabited by approximately 10^{14} mutualistic microbial cells – ten times more cells than the human body consists of (Hooper & Gordon, 2001; Savage, 1977). One gram (wet weight) of the human colon contains about 10^{11} prokaryotes (Whitman *et al.*, 1998). While the gut represents an ideal milieu for bacteria as it is enriched by dietary compounds, bacteria in return help their hosts to digest polysaccharides and provide them with essential vitamins (Conly *et al.*, 1994; Savage, 1986). As babies are born without any germs, their gut is colonized immediately after birth by first mainly facultative and later on mainly obligate anaerobic bacteria to develop a normal healthy digestive tract (Hooper, 2004; Savage, 1977). In fact, exceeding hygiene standards in early childhood can be linked to inflammatory bowel disease (IBD), represented by Crohn´s disease and ulcerative colitis, resulting in intolerance against normal gut inhabitants (Gent *et al.*, 1994). The main colonizers consist largely of two phyla, Bacteroidetes and Firmicutes (Bäckhed *et al.*, 2005; Eckburg *et al.*, 2005), but also include members of the family Enterobacteriaceae. Enterobacteria can be harmless, as it is the case with most types of *Escherichia coli*, but they also harbor pathogens such as *Salmonella typhimurium*, enteropathogenic (EPEC) or enterohemorrhagic *E. coli* (EHEC), which disturb the normal gut flora and cause severe intestinal diseases (Stecher & Hardt, 2008).

While colonizing the gut, bacteria are exposed to substantial changes in the pH range. The stomach contains hydrochloric acid (HCl) and thereby reaches extreme low pH values of 1.5 – 2.5 (Smith, 2003). Furthermore, weak organic acids such as propionic and acetic acids, which are produced during the bacterial fermentation process, lead to additional acidic stress. These weak acids, protonated and uncharged at low pH, can freely diffuse through the cytoplasmic membrane of the bacteria and easily dissociate in the neutral cytoplasm. This leads to decreased internal pH, destabilized inactive macromolecules, and the destruction of the proton motive force (PMF) (Bearson *et al.*, 1997; Foster, 2004; Slonczewski *et al.*, 1981; Zilberstein

et al., 1984). Consequently, bacteria are unable to keep up their proton gradient, which is crucial for ATP formation and cell metabolism. To survive these extreme stress conditions, neutrophil enterobacteria have evolved several passive and active strategies to protect their internal homeostasis (Fig. 1). Passive survival mechanisms include the impermeability of the cytoplasmic membrane for charged ions such as protons and the buffering capacity of the cytoplasm by polyamines, proteins and nucleic acids (Slonczewski & Foster, 1996). By contrast, proton pumps actively export protons out of the cell by consumption of ATP. To avoid further proton influx, other charged ions like potassium are imported by K^+-transporters such as Trk or Kup (Bakker & Mangerich, 1981; Rhoads *et al.*, 1976). Moreover, acidic pH can activate transcription regulators like Fur and PhoP, leading to the synthesis of diverse acid shock proteins (ASPs). These ASPs also contribute to the retention of the internal pH homestasis (Bearson *et al.*, 1997).

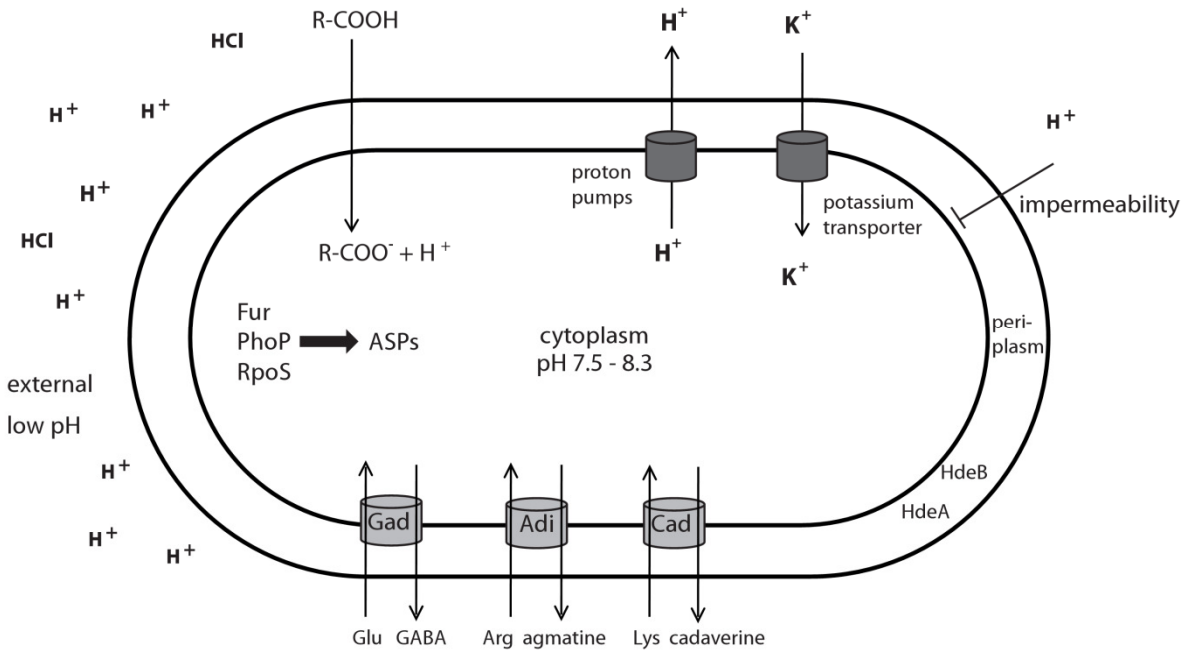


Figure 1: Stress adaptation in *E. coli.* To survive the acidic environment of the gastrointestinal tract, enterobacteria have evolved several different strategies: Passive mechanisms include the impermeability of the cytoplasmic membrane for charged ions and the buffering capacity of the cytoplasm. Proton pumps and potassium transporters belong to the active constitutive mechanisms. Furthermore, the degradative amino acid decarboxylase systems Gad, Adi and Cad are induced by acid stress and glutamate, arginine and lysine, respectively. Their endproducts, gamma aminobutyric acid (GABA), agmatine or cadaverine, are exported out of the cell, thus increasing the external pH. In addition, regulators such as Fur, PhoP and RpoS induce the expression of specific acid shock proteins (ASPs). In the periplasma, chaperones such as HdeA and HdeB protect the proteins from damage.

Additionally, **A**cid **R**esistance systems (AR) contribute to the survival of *E. coli* at a pH of 2 for at least two hours (Foster, 2004; Gorden & Small, 1993). There are five different systems known to help bacteria respond to many different acid stress conditions. In the glucose repressed AR1 system, which is also known as the oxidative system (Bearson *et al.*, 1997), the sigma factor σ^S encoded by the gene *rpoS* and the cAMP receptor protein CRP regulate the expression of several ASPs. The mechanism is still unclear, but AR1 helps cells which were grown to stationary phase in pH 5.5 buffered Lysogeny Broth (LB) medium survive the shift to pH 2.5 buffered minimal medium (Foster, 2004). The systems AR2, AR3 and AR4, also called the Gad (AR2), Adi (AR3) or Cad (AR4) system, require all the activity of an enzyme to decarboxylate the amino acids (aa) glutamate, arginine or lysine, respectively. Within this enzymatic reaction catalyzed by the decarboxylases GadA/GadB, AdiA or CadA, CO_2 is produced and a cellular proton is consumed, thus contributing to an increase of the internal pH. The resulting basic polyamines gamma aminobutyric acid (GABA), agmatine or cadaverine are exported out of the cell by the amino acid/polyamine antiporters GadC, AdiC or CadB, leading to an increase of the extracellular pH (Foster, 2004). AR2, the best known of the AR systems, and AR3 can be partially induced by σ^S and enable cell survival at stationary phase and pH values as low as 2.5 by maintaining an internal pH of 4.2 and 4.7, respectively (Richard & Foster, 2004). By contrast, the pH optimum of the AR4 lysine decarboxylase is higher and lies at pH 5.7 (Sabo *et al.*, 1974). Furthermore, AR3 is only active under anaerobic conditions, while AR4 favors microaerobic conditions. In some *E. coli* strains, but not in K12 strains, the AR5 system exists. This system contains SpeF, an ornithine decarboxylase, and PotE, an ornithine-putrescine antiporter. Like AR4, it functions only under moderately acidic conditions (Applebaum *et al.*, 1977; Zhao & Houry, 2010).

As periplasmic proteins also need acid stress protection, *E. coli* expresses acid inducible chaperones such as HdeA and HdeB, which act in the periplasm by protecting proteins from damage and degradation (Gajiwala & Burley, 2000).

1.2. The Cad system

As mentioned above, *E. coli* possesses the lysine dependent decarboxylase system AR4: the Cad system (Fig. 2). Its main components are the lysine decarboxylase CadA, the lysine/cadaverine antiporter CadB and the regulator of the *cadBA* operon – CadC (Auger *et al.*, 1989; Meng & Bennett, 1992a; Meng & Bennett, 1992b; Sabo *et al.*, 1974; Watson *et al.*, 1992).

1.2.1. CadA, CadB and CadC

In *E. coli*, two different types of amino acid decarboxylases exist: Constitutively expressed biosynthetic decarboxylases important for polyamine synthesis (Goldemberg, 1980; Tabor & Tabor, 1985) and inducible biodegradative decarboxylases like CadA (Gale, 1946). The activity of the cytoplasmic CadA reaches its maximal level when incubated in rich medium containing lysine with low oxygen levels and acidic pH (Sabo *et al.*, 1974). The enzyme catalyzes the decarboxylation of lysine to cadaverine while consuming a proton and releasing CO_2. It has a relative size of 81 kDa (715 aa) and forms decamers consisting of five dimers (Kanjee *et al.*, 2011; Meng & Bennett, 1992a; Sabo *et al.*, 1974). Two CadA decamers and up to five hexamers of the AAA^+-ATPase RavA (ATPases associated with various cellular activities) can build a cage-like complex, which does not influence CadA activity, but stimulates the ATPase activity of RavA (Snider *et al.*, 2006). Furthermore, an interaction with the small alarmone ppGpp blocks CadA activity (Kanjee *et al.*, 2011). Since ppGpp responds to amino acid starvation in the cell, it presumably inhibits the lysine decarboxylase to prevent further lysine consumption.

Following lysine decarboxylation, the end product cadaverine is exported by the lysine/cadaverine antiporter CadB (Meng & Bennett, 1992b). CadB is an inner membrane protein with a relative size of 47 kDa (444 aa) and contains 12 transmembrane helices (Meng & Bennett, 1992a). Furthermore, it shows similarity to the ornithine/putrescine antiporter PotE (Soksawatmaekhin *et al.*, 2004). The exported polyamine cadaverine not only contributes to the increase of the extracellular pH, but also inhibits the activity of the porines OmpC and OmpF, thus decreasing of proton influx (Samartzidou & Delcour, 1999; Samartzidou *et al.*, 2003).

The genes *cadC*, *cadB* and *cadA* are clustered on the *E. coli* chromosome, while *cadB* and *cadA* are organized in an operon containing the same promoter (Auger *et al.*, 1989; Meng & Bennett, 1992a; Watson *et al.*, 1992). The transcriptional regulator CadC activates *cadBA*

expression under low pH conditions and in the presence of lysine (Neely *et al.*, 1994). CadC is anchored in the inner membrane and has a relative molecular mass of 58 kDa (512 aa) (Dell *et al.*, 1994; Watson *et al.*, 1992). It consists of three different domains: the cytoplasmic N-terminal domain (aa 1 - 158), the transmembrane domain (aa 159 - 187) and the periplasmic C-terminal domain (aa 188 - 512) (Dell *et al.*, 1994). CadC belongs to the ToxR-like transcriptional activators, as approximately 100 amino acids of the NH_2-terminus show high similarity to the COOH-termini of other ToxR-like regulators such as ToxR and Tcp of *Vibrio cholera*, PsaE of *Yersinia pseudotuberculosis* and WmpR of *Pseudoalteromonas tunicata* (Egan *et al.*, 2002; Hase & Mekalanos, 1998; Miller *et al.*, 1987; Watson *et al.*, 1992; Yang & Isberg, 1997). Like ToxR, CadC is not part of a two-component-system consisting of a kinase and a response regulator, but rather acts as one-component system with DNA-binding at its NH_2-terminus (Miller *et al.*, 1987; Ulrich *et al.*, 2005; Watson *et al.*, 1992).

1.2.2. Regulation of the Cad system

Several different mechanisms exist to regulate the expression of the *cad* genes *cadA* and *cadB* (Fig. 2). Mutations in the periplasmic domain of CadC revealed that CadC acts as the pH sensor of the system (Dell *et al.*, 1994; Haneburger *et al.*, 2011). In addition, two periplasmic cysteine residues (Cys208 and Cys272) can build a disulfide bridge at neutral pH, which is reduced at low pH. Therefore, cysteines seem to play a functional role in the pH sensing of CadC, too (Tetsch *et al.*, 2011). Detection of the two CadC binding sites Cad1 (-144 to -112 bp) and Cad2 (-89 to -59 bp) in the *cadBA* promoter (P_{cad} or P_{cadBA}), crosslinking-studies and the crystallization of the periplasmic domain led to speculations that CadC binds to the promoter as an oligomer (Eichinger *et al.*, 2011; Küper & Jung, 2005). Furthermore, P_{cadBA} harbors five binding sites for the small histone-like protein H-NS (Küper & Jung, 2005), and also the promoter of CadC is regulated by H-NS (Krin *et al.*, 2010). H-NS binding causes altered DNA topology and transcriptional repression at neutral pH and aerobic conditions (Cortassa & Aon, 1993; Reams *et al.*, 1997; Shi *et al.*, 1993). Anaerobiosis leads to ten times higher CadA activities as aerobiosis (Sabo *et al.*, 1974). In the absence of lysine, the Cad system is inhibited by LysP (Popkin & Maas, 1980; Tabor *et al.*, 1980) - a lysine permease with an average size of 54 kDa (489 aa) consisting of 12 transmembrane domains (Steffes *et al.*, 1992). In the absence of lysine, LysP presumably interacts directly with CadC via its transmembrane domain preventing CadC activation (Tetsch *et al.*, 2008). In the presence of

exogenous lysine, lysine is transported into the cell by LysP leading to reduced interaction with CadC and to *cadBA* expression at acidic pH (Tetsch *et al.*, 2008).

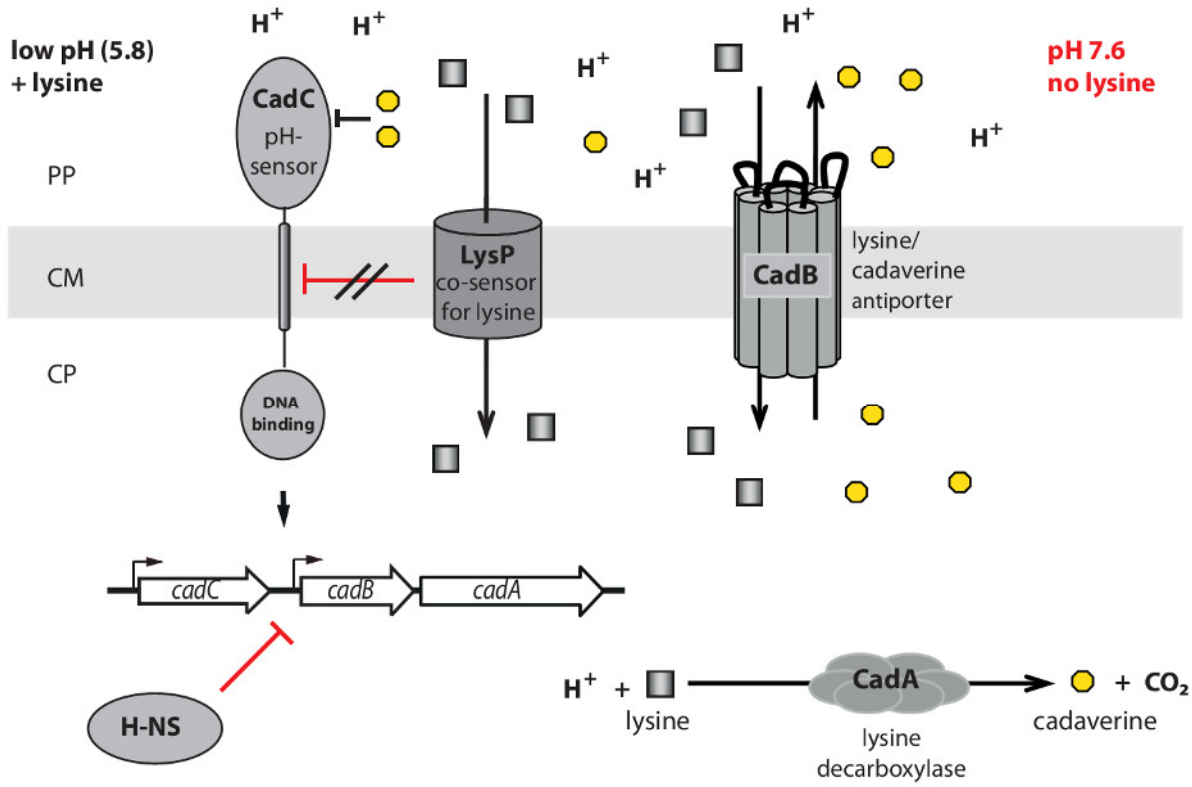


Figure 2: The Cad system of *E. coli*. At neutral pH and in the absence of lysine (in red), the lysine permease LysP inhibits the regulator CadC. Additionally, H-NS prevents *cadBA* expression. A decrease of the external pH, together with the presence of lysine, leads to a release of CadC and lysine transport into the cell by LysP. CadC can bind to the P_{cadBA} promoter, thereby starting the production of the lysine decarboxylase CadA and the antiporter CadB. CadA decarboxylases lysine while consuming a proton and producing CO_2 and cadaverine.

Lysine transport is stimulated at low pH and under anaerobic conditions (Steffes *et al.*, 1992). Moreover, the expression of *lysP* itself is repressed by the incoming lysine and the LysR-type transcriptional regulator ArgP (Neely & Olson, 1996; Ruiz *et al.*, 2011). Interestingly, without lysine ArgP acts as a transcription activator of *lysP* (Ruiz *et al.*, 2011). Another regulatory principle to restrict *cadBA* expression is represented by the negative feedback inhibition of the decarboxylase products cadaverine and CO_2 (Neely *et al.*, 1994; Takayama *et al.*, 1994). Recent studies on the cadaverine inhibition mechanism revealed that the periplasmic domain of CadC binds cadaverine resulting in reduced activity (Haneburger *et al.*, 2012; Tetsch *et al.*,

2008). In addition to these findings, the deletion of the gene *yjeK* resulted in a CadA negative phenotype (Kraxenberger, 2006). YjeK in concert with YjeA has been reported to post-translationally modify the translation elongation factor EF-P (Bailly & de Crecy-Lagard, 2010).

1.3. Steps in translation

Ribosomes translate mRNAs into the corresponding amino acid sequences of proteins (Liljas, 2009). They consist of several rRNAs and ribosomal proteins and make up the 70S complex with the two subunits 30S and 50S in bacteria. In the process of protein synthesis, t-RNA synthetases are needed to ligate amino acids to the 3´CCA-end of their accordant transfer RNAs (tRNAs) (Schimmel, 1987). After the transport of the resulting aminoacyl-tRNAs to the ribosome, a plethora of initiation, elongation and termination factors are recruited to run the protein synthesis. To start translation, the 30S ribosome interacts with the mRNA at its Shine Dalgarno sequence upstream of the start codon AUG [reviewed in (Schmeing & Ramakrishnan, 2009)]. The initiation factors IF-1, IF-2 and IF-3 help the initiator tRNA N-formyl-Met-tRNA (fMet-$tRNA_i$) to localize at the P (peptidyl)-site of the 30S ribosome under GTP consumption by combining the anticodon binding site of the tRNA with the AUG codon on the mRNA (Fig. 3A). Subsequently, the initiation factors are released and the stable 70S complex is built. With the help of elongation factor EF-Tu and GTP consumption, the second amino acid is placed at the A (aminoacyl)-site of the ribosome followed by the formation of the first peptide bond. This step is catalyzed by the peptidyl transferase center of the ribosomal 50S subunit (Fig. 3B). The elongation factor EF-G is then needed to translocate the fMet-$tRNA_i$ to the ribosomal E (exiting)-site and the second amino acid to the ribosomal P-site followed by a release of the fMet-tRNA (Fig. 3C, D). Finally, the termination factors RF-1, RF-2 and RF-3 are important to recognize the stop codon on the mRNA, to release the newly built peptide chain and to dissociate the subunits of the ribosome. In vitro experiments have revealed that the factors mentioned above are not sufficient for proper translation and that additional proteins are needed (Aoki *et al.*, 2008; Ganoza *et al.*, 1985). One of these proteins was assumed to play a significant role in translation initiation: elongation factor EF-P (Glick & Ganoza, 1975).

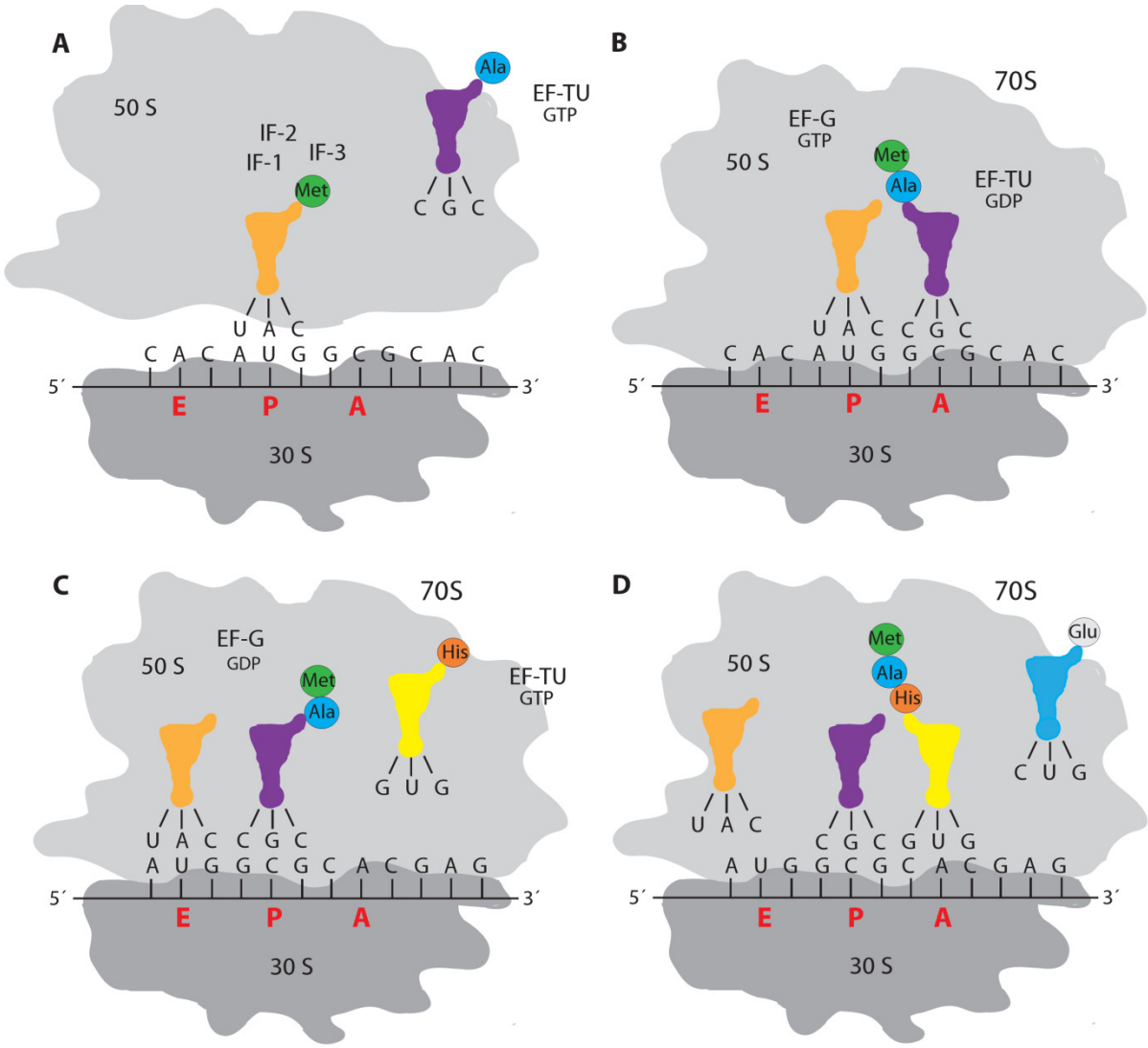


Figure 3: Translation initiation and elongation [reviewed in (Schmeing & Ramakrishnan, 2009)].

A) The initiation factors IF-1, IF-2 and IF-3 help the initiator tRNA N-formyl-Met-tRNA (orange) bind with its anticodon binding site to the start codon AUG, which is localized at the P (peptidyl)-site of the 30S ribosome. After the release of the initiation factors, the 70S complex is formed.

B) The second aminoacyl-tRNA (purple) is placed to the A (aminoacyl)-site of the ribosome by the help of the elongation factor EF-TU. In a peptidyltransferase reaction, the amino acids are linked.

C) The elongation factor EF-G helps the deacetylated initiator tRNA (orange) to translocate to the E (exit)-site**.** The second aminoacyl-tRNA (purple) moves to the P-site. The third aminoacyl-tRNA is shown in yellow.

D) The initiator tRNA (orange) is released from the E-site, and the next aminoacyl-tRNA (yellow) can occupy the A-site. The amino acids are linked by the peptidyltransferase activity of the ribosome. The following aminoacyl-tRNA is shown in blue.

1.4. The elongation factor EF-P

EF-P has been extensively investigated for more than 30 years (Glick & Ganoza, 1975). The protein has a size of 20.5 kDa (187 aa) and is highly conserved in all bacteria (Aoki *et al.*, 1991; Kyrpides & Woese, 1998). As EF-P was suggested to be essential for cell viability, mainly in vitro studies have been performed (Aoki *et al.*, 1997a; Aoki *et al.*, 1997b; Ganoza & Aoki, 2000; Glick *et al.*, 1979). However, subsequent studies described the generation and analysis of *efp* mutants in various bacterial strains (Baba *et al.*, 2006; Kearns *et al.*, 2004; Peng *et al.*, 2001; Zou *et al.*, 2011). The characterization of these mutants revealed the importance of EF-P for swarming and sporulation in *Bacillus subtilis* (Kearns *et al.*, 2004) as well as for virulence and stress response in *Agrobacterium tumefaciens* and *Salmonella enterica* (Peng *et al.*, 2001; Zou *et al.*, 2011). Although EF-P is known to have a stimulatory effect on peptide bond formation between the ribosome-bound initiator transfer RNA fMet-$tRNA_i$ and the tRNA analogue puromycin (Aoki *et al.*, 1991; Glick & Ganoza, 1975), the protein is not an essential component of in vitro translation systems (Shimizu *et al.*, 2001). In *E. coli*, one EF-P copy per ten ribosomes can be found, which resembles the copy number of other initiation factors (An *et al.*, 1980; Cole *et al.*, 1987). However, the detection of EF-P in monosome as well as in polysome fractions led to suggestions that EF-P not only promotes formation of the first peptide bond, but also plays a role during translation elongation (Aoki *et al.*, 2008).

Archaea and eukaryotes possess the orthologous variants aIF-5A and eIF-5A, which share 84% and 64% sequence identity, respectively (Bartig *et al.*, 1992; Cooper *et al.*, 1983). In mammalian cells, the genes encoding eIF-5A1 and its isoform eIF-5A2 are oncogenes (Clement *et al.*, 2003). Furthermore, eIF5a is suggested to play a role in HIV protein Rev mediated transport of mRNAs out of the nucleus, mRNA decay and cell cycle progress (Bevec *et al.*, 1996; Park *et al.*, 1997; Ruhl *et al.*, 1993; Zuk & Jacobson, 1998) as well as in diabetes and malaria (Kaiser, 2012).

eIF5A is unique as it is the only protein known to be post-translationally modified at a conserved lysine residue by hypusine [Nε-(4-amino-2-hydroxybutyl)-lysine] (Cooper *et al.*, 1983), an amino acid derived from lysine and spermidine in two enzymatic steps via the deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) (Abbruzzese *et al.*, 1986; Murphey & Gerner, 1987; Wolff *et al.*, 1995). The activity of eIF5A highly depends on this hypusine modification (Park, 2006; Park *et al.*, 2010; Sasaki *et al.*, 1996). In bacteria, the enzymes DHS and DOHH are missing, and no hypusine has ever been detected. Instead, an

alternative modification has been reported for EF-P (Aoki *et al.*, 2008; Bailly & de Crecy-Lagard, 2010; Navarre *et al.*, 2010; Peil *et al.*, 2012) (Fig. 4).

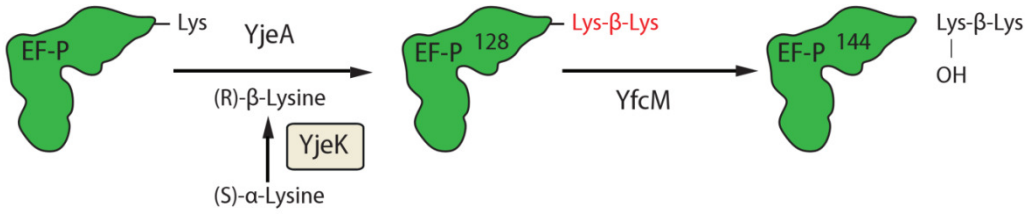


Figure 4: The EF-P modification pathway. YjeK converts (*S*)-α-lysine into (*R*)-β-lysine, which is then transferred to the K34 of *E. coli* EF-P by YjeA and hydroxylated by YfcM. Lysylated EF-P gains an additional mass of 128 Da, hydroxylated EF-P of 144 Da.

The two genes *yjeA* and *yjeK* are often organized in one cluster in bacterial genomes, which has led to the finding that YjeK and YjeA are involved in EF-P modification (Bailly & de Crecy-Lagard, 2010; Yanagisawa *et al.*, 2010). In a first step, the iron sulfur protein YjeK acts as a 2,3-lysine aminomutase (LAM) while catalyzing the isomerisation of (S)-α-lysine to (R)-β-lysine (Behshad *et al.*, 2006). The (R)-β-lysine is then activated and transferred to the conserved lysine residue 34 (K34) of EF-P by YjeA resulting in a modified and thus active EF-P (Gilreath *et al.*, 2011; Roy *et al.*, 2011). YjeA (also known as PoxA or GenX) is a homolog to the class II lysyl-tRNA-synthetase LysRS. In *E. coli*, YjeA shows 32% identity and 50% homology to the COOH-terminus of LysRS containing the catalytic core important for lysine binding, but it lacks the NH_2-terminus of LysRS with the anticodon binding site important for tRNA binding (Ambrogelly *et al.*, 2010; Navarre *et al.*, 2010). Therefore, it has been suggested that YjeA has a function differing from the aminoacylation of tRNAs (Bailly & de Crecy-Lagard, 2010). Drawing on mass spectrometry (MS) analysis for modified EF-P (Aoki *et al.*, 2008), Peil *et al.* have reported recently that K34 of EF-P is additionally hydroxylated by the O_2 dependent hydroxylase YfcM (Peil *et al.*, 2012) (Fig. 4). However, a deletion of *yfcM* leads to a wildtype like phenotype in *Salmonella enterica*, which indicates that the hydroxylation plays a minor role in vivo (Bullwinkle *et al.*, 2013).

Crystal structures of bacterial EF-P reveal a three-domain (I-III) architecture resembling the L-shape of tRNAs (Blaha *et al.*, 2009; Hanawa-Suetsugu *et al.*, 2004; Yanagisawa *et al.*, 2010). It has been assumed that modified EF-P interacts with the 3´CCA-end of tRNAs bound

to the P-site of the ribosome (P-tRNAs) and stimulates peptide bond formation through stabilization and positioning of the acceptor end of the P-tRNAs (Blaha *et al.*, 2009). In addition, several further functions of EF-P have been suggested, including the stimulation of the first peptide bond (Glick *et al.*, 1979), the coupling of the ribosomal 30S and 50S subunits (Aoki *et al.*, 1991), the enhancement of polylysine synthesis (Glick & Ganoza, 1976), the change of the conformation of the ribosomal active site (Ganoza & Aoki, 2000) and the inhibition of tRNAs to bind to the A-site before ribosomal proofreading (Aoki *et al.*, 2008). As the overall protein synthesis is diminished by only 30% in yeast cells, it has been assumed that eIF5A does not play a general role in the translational machinery, but rather has importance only for a small subset of mRNAs (Kang & Hershey, 1994). For example, the translation of the porin KdgM is dependent on EF-P in *Salmonella*, thus a deletion of *efp* results in increased membrane permeability and in various phenotypes like attenuated virulence in the bacterium (Zou *et al.*, 2011). Although plenty suggestions have been made for the action mode of EF-P, in vivo data are still missing.

1.5. Scope of the thesis

In this study, the relevance of elongation factor P and its modifying proteins YjeK and YjeA in regard to the Cad system is analyzed in more detail. To this end, *yjeA*/*yjeK* and *efp* mutants are characterized by determining their lysine decarboxylase phenotype, their capability for *cadBA* expression under non- and inducing conditions and their ability to interact with each other and other components of the Cad system.

Additionally it is examined, which of the Cad proteins is dependent on EF-P mediated translation. As no direct EF-P - target has ever been identified before in *E. coli*, the finding of the first target can greatly improve the understanding of the function of EF-P.

Furthermore it is investigated, if EF-P recognizes a special signal sequence. With the signal sequence in hand, further targets can be identified. As the physiological role of EF-P is still unclear, the signal sequence can provide first insights into the mode of action of EF-P in vivo.

To confirm the importance of EF-P for the translation of target proteins, in vitro translation assays are performed by Dr. Daniel Wilson and Dr. Agata Starosta (Gene Center, LMU Munich).

Finally, the effect of a *hfq* deletion on *cadBA* transcription and on expression of CadC, YjeA, YjeK and EF-P is investigated in more detail.

2. Materials and Methods

2.1. Materials

Following materials were used in this study:

Table 1: Materials and their manufacturers used in this study.

Material	Manufacturer
[γ-^{32}P] ATP	Perkin Elmer (Rodgau)
5-Bromo-4-Chloro-3-Indoxyl Phosphate (BCIP)	AppliChem (Darmstadt)
Acrylamide Protogel	Biozym Diagnostik (Hessisch Oldendorf)
Agarose	Serva (Heidelberg)
Alkaline Phosphatase	New England Biolabs (Frankfurt)
Conjugated anti-Mouse IgG	GE Healthcare (Braunschweig)
Ammonium Persulfate (APS)	National Diagnostics (Atlanta)
Ampicillin (sodium salt)	Roth (Karlsruhe)
Anti-Rabbit IgG	GE Healthcare(Braunschweig)
Calf Intestinal Phosphatase (CIP)	New England Biolabs (Frankfurt)
Carbenicillin (disodium salt)	Roth (Karlsruhe)
Chloramphenicol	Sigma (Deisenhofen)
Chloroform	Roth (Karlsruhe)
Diethylpyrocarbonate (DEPC)	Roth (Karlsruhe)
DNA oligonucleotides	Sigma (Deisenhofen) Invitrogen (Karlsruhe) Eurofins MWG Operon (Ebersberg)
DNase I	Sigma (Deisenhofen)
DNA standard (2-Log DNA-Ladder)	New England Biolabs (Frankfurt)
DNeasy 96 blood and tissue kit	Qiagen (Hilden)
dNTPs (deoxynucleotide triphosphates)	Invitrogen (Karlsruhe)
Glycerol	Roth (Karlsruhe)
Hi Yield PCR Clean-Up & Gel-Extraction Kit	Süd-Laborbedarf (Gauting)

Hi Yield Plasmid Mini Kit	Süd-Laborbedarf (Gauting)
Hybond-P protein transfer membrane	GE Healthcare (Braunschweig)
Isopropyl-D-thiogalactopyranoside (IPTG)	PeqLab (Erlangen)
Kanamycin (sulfate)	Roth (Karlsruhe)
L-(+) - Arabinose	Roth (Karlsruhe)
L-Lysine	Sigma (Deisenhofen)
Nitro Blue Tetrazolium (NBT)	Biomol (Hamburg)
Ni-NTA agarose	Qiagen (Hilden)
Ni-NTA magnetic agarose beads	Qiagen (Hilden)
Nitrocellulose membrane	GE Healthcare (Braunschweig)
Page Ruler Prestained Protein Ladder Plus	Fermentas (St. Leon-Rot)
Penta-His anti-Mouse-IgG	Qiagen (Hilden)
Phenol-Chloroform	Roth (Karlsruhe)
Phenol/Chloroform/Isoamylalcohol (25/24/1)	Roth (Karlsruhe)
Phenylmethylsulfonyl fluoride (PMSF)	Sigma (Deisenhofen)
Phusion DNA Polymerase	Finnzyme (Espoo, Finnland)
Pyridoxal Phosphate (PLP)	Fluka (Neu-Ulm)
Quick & Easy *E. coli* Gene Deletion Kit	Gene Bridges (Heidelberg)
Restriction Nuclease	New England Biolabs (Frankfurt)
Bovine Serum Albumin (BSA)	AppliChem (Darmstadt)
Spermidine	Fluka (Neu-Ulm)
T4 DNA Ligase	New England Biolabs (Frankfurt)
Taq-DNA Polymerase	Peqlab (Erlangen)
Thermoscript Reverse Transcriptase	Invitrogen (Karlsruhe)
Trinitrobenzenesulfonic acid (TNBS)	Sigma (Deisenhofen)
Triton X-100	Calbiochem (La Jolla, Kalifornien)
Tween 20	GE Healthcare (Braunschweig)

All materials not listed here were purchased from the manufacturers Bayer (Leverkusen), Biomol (Hamburg), Biorad (Munich), Biozym Diagnostics GmbH (Hess, Oldendorf), Fluka (Neu-Ulm), Gibco/BRL (Eggenstein), ICN Biomedicals Inc. (Aurora, Ohio), Merck (Darmstadt), Riedel-de Häen (Seelze), Roche Diagnostics (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen) with the purity grade „pro analysis“.

2.2. Strains, plasmids and oligonucleotides

Strains used in this study are listed in Table 2, plasmids in Table 3 and oligonucleotides in Table 4.

Table 2: Strains used in this study. All strains are *Escherichia coli* derivates.

Strain	Feature	Source
DH5α	F⁻ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	Promega
MG1655	*E. coli* K-12 reference strain	(Blattner *et al.*, 1997)
P1#36	MG1655 $yjeK_{643\text{-}1029}$::Tn*10*	(Kraxenberger, 2006)
TK1	MG1655 $yjeK_{643\text{-}1029}$*::npt*	(Kraxenberger, 2006)
BW25113	Δ*(araD-araB)567*, Δ*lacZ4787*(::*rrnB-3*), lambda⁻, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*	(Baba *et al.*, 2006)
JW4116	BW25113 *yjeA*::*npt*	(Baba *et al.*, 2006)
JW4106	BW25113 *efp*::*npt*	(Baba *et al.*, 2006)
JW4094	BW25113 *cadC*::*npt*	(Baba *et al.*, 2006)
MG-CL-1	MG1655 Δ*lacZ*::*tet rpsL*150 Δ*cadA cadA*::*lacZ*	this study
MG-CR	MG1655 Δ*lacZ*::*tet rpsL*150 Δ*cadBA cadBA*::*lacZ*	(Ruiz *et al.*, 2011)
MG-CL 1-3	MG1655 Δ*lacZ*::*tet rpsL*150 *cadC::npt* Δ*cadCBA cadBA*::*lacZ*	(Schüppel, 2010)
MG-CL-12-*yjeA*	MG1655 Δ*lacZ*::*tet rpsL*150 *yjeA::npt* Δ*cadBA cadBA*::*lacZ*	this study
MG-CL-12-*yjeK*	MG1655 Δ*lacZ*::*tet rpsL*150 $yjeK_{642\text{-}1029}$*::npt* Δ*cadBA cadBA*::*lacZ*	this study
MG-CL-12-*hflx*	MG1655 Δ*lacZ*::*tet rpsL*150 $hflx_{1\text{-}750}$*::npt* Δ*cadBA cadBA*::*lacZ*	this study
MG-CL-12-*hfq*	MG1655 Δ*lacZ*::*tet* $hfq_{1\text{-}230}$*::cat* Δ*cadBA cadBA*::*lacZ*	this study
SU1	BW25113 $yjeK_{643\text{-}1029}$*::cat*	this study
BL21 (DE3)	*E. coli* B F⁻*dcm ompT hsdS* ($r_B^-m_B^-$) *gal*	(Studier & Moffatt, 1986)
SU2	MG1655 *yjeA*::*npt*	this study
SU3	BL21 (DE3) $yjeK_{643\text{-}1029}$*::cat*	this study
SU4	JW4106 *hns::cat*	this study
AM111	MC4100 Δ*hfq*	(Muffler *et al.*, 1996)

Table 3: Plasmids used in this study. Plasmid constructions are further explained in the middle column.

Plasmid	Feature / Construction comments	Source
General vectors		
pET16b	Amp^r-cassette, T7-promoter, pBBR322 origin, 5'-His-tag coding sequence, *lacI*-coding sequence, *lac* operator	Novagen (Merck Millipore)
pBAD24	Amp^r-cassette, pBBR322 origin, *araC* coding sequence, *ara* operator	(Guzman *et al.*, 1995)
pBBR1-MCS5	Gm^r-cassette, pBBR broad host range origin of replication, mob region for conjugative transfer	(Kovach *et al.*, 1995)
pBBR1-MCS3-LacZ	Tet^r-cassette, pBBR broad host range origin of replication, mob region for conjugative transfer, *lacZ* coding sequence	(Fried *et al.*, 2012)
pQE70	Amp^r-cassette, 5'-His-Tag coding sequence, *lacI*-coding sequence, *lac* operator	Qiagen
pIVEX2.3MCS	Amp^r-cassette, T7-promoter, 3'-His-Tag coding sequence	Roche
pQE70-Eco_efp	*efp* cloned using SphI, BamHI restrictions sites	(Peil *et al.*, 2012)
pQE70-Eco_efp K34A/	pQE70 plasmid harboring *efp* with a single mutation (K34A)	Dr. Agata Starosta
pQE70-Eco_efp K34R	pQE70 plasmid harboring *efp* with a single mutation (K34R)	Dr. Agata Starosta
pAF/*kamA*	Amp^r-cassette,T7 promoter, pET23a origin	(Ruzicka *et al.*, 2000)
pAlter-Ex2/*argU*	Tet^r-cassette, *tac* promoter, p15a origin	(Ruzicka *et al.*, 2000)
pET16b-His_6-YjeK	*yjeK* in pET16b, 5´-His_6-tag	(Kraxenberger, 2006)
pBAD24-*yjeA*	*yjeA* in pBAD24, with NdeI and XbaI, 5´-His_6-tag, P121+P122	this study
pBAD24-*yjeK*	pBAD24 with NcoI-XmaI fragment from pUC19-rbs-his_{10}-*yjeK,* 5´-His-tag	(Kraxenberger, 2006)
pBAD24-*hfq*	*hfq* in pBAD24, with NdeI and EcoRI, P119+P120, 3´-His-tag	this study
***cadC-lacZ* promoter fusions**		
p3LC-TF	Fusion of *cadC* promoter with artificial RBS to *lacZ* / P1+P3 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pst*I)	this study
p3LC-TL02	Fusion of *cadC* promoter to sequence encoding 2 amino acids of *cadC* and *lacZ*/ P1+P4 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL02 (Ala, Gly, His, Leu, Met, Phe, Pro)	*cadC* promoter fusion with artificial RBS to *lacZ*+variabel second aa (Ala, Gly, His, Leu, Met, Phe or Pro)/P1+P111-P117 PCR fragments in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30	Translational CadC'-LacZ fusion (sequence encodes 30 amino acids of *cadC*)/ P1+P5 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL66	Translational CadC'-LacZ (sequence encodes 66 amino acids of *cadC*)/ P1+P6 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL100	Translational CadC'-LacZ fusion (sequence encodes 100 amino acids of *cadC*)/ P1+P7 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL108	Translational CadC'-LacZ fusion (sequence encodes 108 amino acids of *cadC*)/ P1+P8 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL131	Translational CadC'-LacZ fusion (sequence encodes 131 amino acids of *cadC*)/ P1+P9 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL158	Translational CadC'-LacZ fusion (sequence encodes 158 amino acids of *cadC*)/ P1+P10 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I+*Pci*I/*Nco*I)	this study
p3LC -TL158-CSPPP	Translational CadC´-LacZ fusion; (sequence encodes 158 amino acids of *cadC*), S118C substitution in *cadC*, P11+12 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study

Plasmid	Feature / Construction comments	Source
p3LC -TL158-GSPPP	Translational CadC´-LacZ fusion; (sequence encodes 158 amino acids of *cadC*), S118G substitution in *cadC*, P13+P14 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL30-GPP	Translational CadC´-LacZ fusion; sequence encodes 30 amino acids of *cadC*+sequence encoding GPP, P1+P15 fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL30-PGP	Translational CadC´-LacZ fusion; (sequence encodes 30 amino acids of *cadC*+sequence encoding PGP, P1+P16 fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL30-PPG	Translational CadC´-LacZ fusion; (sequence encodes 30 amino acids of *cadC*+sequence encoding PPG, P1+P17 fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Artificial introduction of three consecutive prolines		
p3LC-TL02-P	p3LC-TL02 + sequence encoding 1 proline / P1+P18 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL02-3P	p3LC-TL02+ sequence encoding 3 prolines / P1+P19 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-3P	p3LC-TL30 + sequence encoding 3 prolines / P1+P20 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL66-3P	p3LC-TL66 + sequence encoding 3 prolines / P1+P21 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL100-3P	p3LC-TL100 + sequence encoding 3 prolines / P1+P22 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL108-3P	p3LC-TL108 + sequence encoding 3 prolines / P1+P23 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Artificial modification of the proline stretch length in CadC´-LacZ TL30		
p3LC-TL30-5P	p3LC-TL30 + sequence encoding 5 prolines / P1+P24 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-4P	p3LC-TL30 + sequence encoding 4 prolines / P1+P25 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-2P	p3LC-TL30 + sequence encoding 2 prolines / P1+P26 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-1P	p3LC-TL30 + sequence encoding 1 proline / P1+P27 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Artificial modification of the proline stretch codon usage		
p3LC-TL30-3P-CCA	p3LC-TL30 + sequence for 3 x CCA / P1+P28 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-3P-CCG	p3LC-TL30 + sequence for 3 x CCG / P1+P29 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-3P-CCT	p3LC-TL30 + sequence for 3 x CCT / P1+P30 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Artificial introduction of sequences encoding stretches of various amino acids		
p3LC-TL30-5F	p3LC-TL30 + sequence encoding 5 phenylalanines / P1+P31 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5G	p3LC-TL30 + sequence encoding 5 glycines / P1+P32 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5A	p3LC-TL30 + sequence encoding 5 alanines / P1+P33 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5V	p3LC-TL30 + sequence encoding 5 valines / P1+P34 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5L	p3LC-TL30 + sequence encoding 5 leucines / P1+P35 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5I	p3LC-TL30 + sequence encoding 5 isoleucines / P1+P36 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study

Plasmid	Feature / Construction comments	Source
p3LC-TL30-5M	p3LC-TL30 + sequence encoding 5 methionines / P1+P37 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5S	p3LC-TL30 + sequence encoding 5 serines / P1+P38 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5T	p3LC-TL30 + sequence encoding 5 threonines / P1+P39 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5D	p3LC-TL30 + sequence encoding 5 aspartates / P1+P40 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5E	p3LC-TL30 + sequence encoding 5 glutamates / P1+P41 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5Y	p3LC-TL30 + sequence encoding 5 tyrosines / P1+P42 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5N	p3LC-TL30 + sequence encoding 5 asparagines / P1+P43 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5K	p3LC-TL30 + sequence encoding 5 lysines / P1+P44 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5H	p3LC-TL30 + sequence encoding 5 histidines / P1+P45 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5Q	p3LC-TL30 + sequence encoding 5 glutamines / P1+P46 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5C	p3LC-TL30 + sequence encoding 5 cysteines / P1+P47 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5W	p3LC-TL30 + sequence encoding 5 tryptophans / P1+P48 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-TL30-5R	p3LC-TL30 + sequence encoding 5 arginines / P+P49 PCR fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Generation of CadC Pro to Ala substitution mutants		
p5C	*cadC* native copy including the native promoter / P1+P2 PCR fragment in pBBR1-MCS5 (*Xba*I +*PspOM*I)	this study
p5C-P120A	*cadC* mutant derivative P120A including the native promoter / P50+P51 PCR-OL-fragment in pBBR1-MCS5 (*Xba*I +*PspOM*I)	this study
p5C-P121A	*cadC* mutant derivative P121A including the native promoter / P52+P53 PCR-OL-fragment in pBBR1-MCS5 (*Xba*I +*PspOM*I)	this study
p5C-P122A	*cadC* mutant derivative P122A including the native promoter / P54+P55 PCR-OL-fragment in pBBR1-MCS5 (*Xba*I +*PspOM*I)	this study
p5C-P/A	*cadC* mutant derivative PPPIP/AAAIS including the native promoter / P56+P57 PCR-OL-fragment in pBBR1-MCS5 (*Xba*I +*PspOM*I)	this study
p3LC -TL158-P120A	p3LC-TL158 mutant derivative P121A / P1+P10 PCR fragment from p5C-P120A template in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL158-P121A	p3LC-TL158 mutant derivative P121A / P1+P10 PCR fragment from p5C-P121A template in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL158-P122A	p3LC-TL158 mutant derivative P121A / P1+P10 PCR fragment from p5C-P122A template in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC -TL158-P/A	p3LC-TL158 mutant derivative PPPIP/AAAIS / P1+P10 PCR fragment from p5C-P/A template in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
pET16b-CadC	*cadC* native copy under control of IPTG inducible T7 polymerase dependent promote, His_6-CadC	(Küper & Jung, 2005)
pET16b-CadC-P/A	*cadC* mutant derivative PPPIP/AAAIS T7 polymerase dependent / PCR fragment from p5C-P/A template in pET16b	this study
Translational LacZ fusions of other EF-P target proteins fused to the CadC native promoter		
p3LC-cadA	Translational CadA-LacZ fusion including complete *cadA* but replacing the *cadAB* promoter by the *cadC* promoter / 61+62 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*BspHI*/*Nco*I)	this study
p3LC-cadB	Translational CadB-LacZ fusion including the complete *cadB* but replacing the *cadAB* promoter by the *cadC* promoter / 58+59 PCR-OL-Fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study

Plasmid	Feature / Construction comments	Source
p3LC-flk	Translational FlK-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-amiB	Translational AmiB-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-flhC	Translational FlhC-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-nlpD	Translational NlpD-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-rzoR	Translational RzoR-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-tonB	Translational TonB-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-uvrB	Translational UvrB-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-hisD	Translational HisD-LacZ fusion; for construction see table 6 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-flhC-APP	Translational FlhC-LacZ fusion; P/A substitute, P83-P84 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-flhC-PAP	Translational FlhC-LacZ fusion; P/A substitute, P85+P86 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
p3LC-flhC-AAA	Translational FlhC-LacZ fusion; P/A substitute, P87+P88 PCR-OL-fragment in pBBR1-MCS3-LacZ (*Xba*I +*Pci*I/*Nco*I)	this study
Bacterial two hybrid studies		
pKT25	*ori*-p15a T25 fragment for C-terminal fusion; Km^r	Euromedex
pKNT25	*ori*-p15a T25 fragment for N-terminal fusion; Km^r	Euromedex
pUT18	*ori*-ColE1 T18 fragment for N-terminal fusion; Amp^r	Euromedex
pUT18C	*ori*-ColE1 T18 fragment for N-terminal fusion; Amp^r	Euromedex
pUT18-zip	Positive control plasmid; encoding for yeast leucine zipper GCN4	Euromedex
pKT25-zip	Positive control plasmid; encoding for yeast leucine zipper GCN4	Euromedex
pKT25-CadC	*cadC* in pKT25 (*Xba*I+*BamH*I), P166+P167	(Krönauer, 2011)
pKT25-LysP	*lysP* in pKT25, (*Xba*I+*BamH*I), P168+P169	(Krönauer, 2011)
pKT25-YjeA	*yjeA* in pKT25, (*Xba*I+*BamH*I), P170+P171	(Krönauer, 2011)
pKT25-YjeK	*yjeK* in pKT25, (*Xba*I+*BamH*I), P174+P175	(Krönauer, 2011)
pKT25-EFP	*efp* in pKT25, (*Xba*I+*BamH*I), P164+P165	(Krönauer, 2011)
pKT25-HNS	*hns* in pKT25, (*Xba*I+*BamH*I), P172+P173	(Krönauer, 2011)
pKTN25-CadC	*cadC* in pKTN25, (*Xba*I+*BamH*I), P166+P167	(Krönauer, 2011)
pKTN25-LysP	*lysP* in pKTN25, (*Xba*I+*BamH*I), P168+P169	(Krönauer, 2011)
pKTN25-YjeA	*yjeA* in pKTN25, (*Xba*I+*BamH*I), P170+P171	(Krönauer, 2011)
pKTN25-YjeK	*yjeK* in pKTN25, (*Xba*I+*BamH*I), P174+P175	(Krönauer, 2011)
pKTN25-EFP	*efp* in pKTN25, (*Xba*I+*BamH*I), P164+P165	(Krönauer, 2011)
pKTN25-HNS	*hns* in pKTN25, (*Xba*I+*BamH*I), P172+P173	(Krönauer, 2011)
pUT18C-CadC	*cadC* in pUT18C, (*Xba*I+*BamH*I), P166+P167	(Krönauer, 2011)
pUT18C-LysP	*lysP* in pUT18C, (*Xba*I+*BamH*I), P168+P169	(Krönauer, 2011)

Plasmid	Feature / Construction comments	Source
pUT18C-YjeA	*yjeA* in pUT18C, (*Xba*I+*BamH*I), P170+P171	(Krönauer, 2011)
pUT18C-YjeK	*yjeK* in pUT18C, (*Xba*I+*BamH*I), P174+P175	(Krönauer, 2011)
pUT18C-EFP	*efp* in pUT18C, (*Xba*I+*BamH*I), P164+P165	(Krönauer, 2011)
pUT18C-HNS	*hns* in pUT18C, (*Xba*I+*BamH*I), P172+P173	(Krönauer, 2011)
Generation of CadC Ly to Ala mutants		
pET16b-K59A	*cadC* mutant derivate K59A under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K77A	*cadC* mutant derivate K77A under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K95A	*cadC* mutant derivate K95A under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K99A	*cadC* mutant derivate K99A under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K80A	*cadC* mutant derivate K80A under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
Generation of CadC Ly to Arg mutants		
pET16b-K59R	*cadC* mutant derivate K59R under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K77R	*cadC* mutant derivate K77R under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K95R	*cadC* mutant derivate K95R under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K99R	*cadC* mutant derivate K99R under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
pET16b-K80R	*cadC* mutant derivate K80R under control of IPTG inducible T7 polymerase dependent promoter/PCR-OL-fragment in pET16b-CadC	this study
P_{cadA}-*lacZ* fusions		
P3C-PrA79	pBBR1-MCS3-LacZ harboring 79 bp of the sequence upstream of *cadA* +1, P200+P209	this study
P3C-PrA100	pBBR1-MCS3-LacZ harboring 100 bp of the sequence upstream of *cadA* +1, P201+P209	this study
P3C-PrA200	pBBR1-MCS3-LacZ harboring 200 bp of the sequence upstream of *cadA* +1, P202+P209	this study
P3C-PrA300	pBBR1-MCS3-LacZ harboring 300 bp of the sequence upstream of *cadA* +1, P203+P209	this study
P3C-PrA400	pBBR1-MCS3-LacZ harboring 400 bp of the sequence upstream of *cadA* +1, P204+P209	this study
P3C-PrA500	pBBR1-MCS3-LacZ harboring 500 bp of the sequence upstream of *cadA* +1, P205+P209	this study
P3C-PrA600	pBBR1-MCS3-LacZ harboring 600 bp of the sequence upstream of *cadA* +1, P206+P209	this study
P3C-PrA700	pBBR1-MCS3-LacZ harboring 700 bp of the sequence upstream of *cadA* +1, P207+P209	this study
P3C-PrA800	pBBR1-MCS3-LacZ harboring 800 bp of the sequence upstream of *cadA* +1, P208+P209	this study

Table 4: Oligonucleotides used in this study. All primers were purchased from Sigma Aldrich and diluted in 1xTE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) for storage at -20°C and -80°C.

	Name	Sequence	Restric-tion site	Source
***cadC-lacZ* promoter fusions**				
P1	XbaI-CadC-NP-Fw	AGT CTA GAC CTG AGC TAT AGC ACT AAC TGA CG	*Xba*I	this study
P2	PspOMI-CadC-Rev	CCG GGC CCA AAC TCA ACA ACA AAT ATT TCC GAG CAT A	*PspOM*I	this study
P3	PstI-CadC-TriF-Rev	GCC TGC AGA ATA GAA ACT CAT TCG AAA AGG GAA TGA TG	*PstI*	this study
P4	PciI-CadC-TF02-Gln-Rev	GCA CAT GTG TTG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P5	PciI-TF30E-CadC-Rev	GCA TAC ATG TGC TCA AGG GTA AGT TGA CGC CCA TTG CG	*Pci*I	this study
P6	PciI-TF66-CadC-Rev	GCA CAT GTG ATT GGT GAC AAT ACT TCT CTT CCA G	*Pci*I	this study
P7	PciI-TF100-CadC-Rev	GCA CAT GTT TAA TTT ATA GCC GCG CTT TGG TAC A	*Pci*I	this study
P8	PciI-CadC-LacZ-TL108-Rev	CGA CAT GTG GCT GTA CCA GAT AAC CGG CAC C	*Pci*I	this study
P9	PciI-TF131-CadC-Rev	GAA CAT GTC TGT GGC AGG AAC CGC CTC	*Pci*I	this study
P10	PciI-TF158-CadC-Rev	GCA CAT GTA GGT AGT GAA TCG TTT GCT TTT AAC TGG	*Pci*I	this study
P11	CadC-CSPPP-fw	GAA ATA ATG CTA TGT TCG CCT CCC		this study
P12	CadC-CSPPP-rev	GGG AGG CGA ACA TAG CAT TAT TTC		this study
P13	CadC-GSPPP-fw	GGA AAT AAT GCT AGG TTC GCC TCC		this study
P14	CadC-GSPPP-rev	GGA GGC GAA CCT AGC ATT ATT TCC		this study
P15	PciI-TL30-CadC-GPP-Rev	GTA CAT GTG CGG CGG GCC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P16	PciI-TL30-CadC-PGP-Rev	GTA CAT GTG CGG GCC CGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P17	PciI-TL30-CadC-PPG-Rev	GTA CAT GTG GCC CGG CGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
Artificial introduction of three consecutive prolines				
P18	PciI-CadC-TF02-Pro-Rev	GCA CAT GTG CGG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P19	PciI-CadC-TF02-3xPro-Rev	GCA CAT GTG TGG TGG AGG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P20	PciI-TF30-CadC-3Pro-Rev	GTA CAT GTG TGG TGG AGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P21	PciI-TF66-CadC-3xPro-Rev	GCA CAT GTG TGG TGG AGG ATT GGT GAC AAT ACT TCT CTT CCA G	*Pci*I	this study
P22	PciI-TF100-CadC-3xPro-Rev	GCA CAT GTT TGG TGG AGG TAA TTT ATA GCC GCG CTT TGG TAC A	*Pci*I	this study
P23	PciI-CadC-LacZ-TL108-3xPro-Rev	GCA CAT GTG TGG TGG AGG GCT GTA CCA GAT AAC CGG CAC C	*Pci*I	this study

	Name	Sequence	Restric-tion site	Source
Artificial modification of the proline stretch length in CadC´-LacZ TL30				
P24	PciI-TF30-CadC-5Pro-Rev	GTA CAT GTG TGG TGG AGG TGG TGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P25	PciI-TF30-CadC-4Pro-Rev	GTA CAT GTG TGG TGG AGG TGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P26	PciI-TF30-CadC-2Pro-Rev	GTA CAT GTG TGG TGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P27	PciI-TF30-CadC-1Pro-Rev	GTA CAT GTG TGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
Artificial modification of the proline stretch codon usage				
P28	PciI-TF30-CadC-3Pro-CCA-Rev	GTA CAT GTG TGG TGGTGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P29	PciI-TF30-CadC-3Pro-CCT-Rev	GTA CAT GTG AGG AGGAGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P30	PciI-TF30-CadC-3Pro-CCC-Rev	GTA CAT GTG GGG GGGGGG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
Artificial introduction of stretches of all other amino acids				
P31	PciI-TF30-CadC-5Phe-Rev	GTA CAT GTG GAA GAA AAA GAA GAA CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P32	PciI-TF30-CadC-5Gly-Rev	GTA CAT GTG ACC GCC ACC GCC ACC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P33	PciI-TF030-CadC-5Ala-Rev	GTA CAT GTG CGC GGC CGC GGC CGC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P34	PciI-TF30-CadC-5Val-Rev	GTA CAT GTG CAC AAC CAC AAC CAC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P35	PciI-TF30-CadC-5Leu-Rev	GTA CAT GTG CAG CAGCAGCAGCAG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P36	PciI-TF30-CadC-5Ile-Rev	GTA CAT GTG AAT GAT AAT GAT AAT CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P37	PciI-TF30-CadC-5Met-Rev	GTA CAT GTG CAT CATCATCATCAT CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P38	PciI-TF30-CadC-5Ser-Rev	GTA CAT GTG CGA CGA TGA CGA TGA CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P39	PciI-TF30-CadC-5Thr-Rev	GTA CAT GTG GGT CGT GGT CGT GGT CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P40	PciI-TF30-CadC-5Asp-Rev	GTA CAT GTG ATC GTC ATC GTC ATC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P41	PciI-TF30-CadC-5Glu-Rev	GTA CAT GTG CTC TTC CTC TTC CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P42	PciI-TF30-CadC-5Tyr-Rev	GTA CAT GTG ATA GTA ATA GTA ATA CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study

	Name	Sequence	Restric-tion site	Source
P43	PciI-TF30-CadC-5Asn-Rev	GTA CAT GTG ATT GTT ATT GTT ATT CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P44	PciI-TF30-CadC-5Lys-Rev	GTA CAT GTG TTT CTT TTT CTT TTT CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P45	PciI-TF30-CadC-5His-Rev	GTA CAT GTG ATG GTG ATG GTG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P46	PciI-TF30-CadC-5Gln-Rev	GTA CAT GTG CTG TTG CTG TTG CTG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P47	PciI-TF30-CadC-5Cys-Rev	GTA CAT GTG GCA ACA GCA ACA GCA CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P48	PciI-TF30-CadC-5Trp-Rev	GTA CAT GTG CCA CCACCACCACCA CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
P49	PciI-TF30-CadC-5Arg-Rev	GTA CAT GTG GCG ACG GCG ACG GCG CTC AAG GGT AAG TTG ACG CCC ATT GCG	*Pci*I	this study
Generation of CadC Pro to Ala substitution mutants				
P50	CadC-OL-120A-fw	TGC TAT CTT CGG CTC CCC CTA TAC C		this study
P51	CadC-OL-120A-rev	GGT ATA GGG GGA GCC GAA GAT AGC A		this study
P52	CadC-P121A-Whobble-fw	CTA TCT TCG CCT GCC CCT ATA CCA G		this study
P53	CadC-P121A-Whobble-rev	GGT ATA GGG GGA GCC GAA GAT AGC A		this study
P54	CadC-OL-122A-fw	TCT TCG CCT CCC GCT ATA CCA GAG		this study
P55	CadC-OL-122A-rev	CTC TGG TAT AGC GGG AGG CGA AGA		this study
P56	CadC(Pro/Ala)-OL-fw	GCG GCGGCG ATA TCG GAG GCG GTT CCT GCC ACA G		this study
P57	CadC(Pro/Ala)-OL-rev	CTG TGG CAG GAA CCG CCT CCG ATA TCG CCG CCG C		this study
Translational LacZ fusions of other EF-P target proteins fused to the CadC native promoter				
P58	NP-CadC-cadB-OL-Rev	GCA GAA CTC ATA ATA GAA ACT CAT TCG AAA AGG GAA TGA TG		this study
P59	NP-CadC-cadB-OL-Fw	GAG TTT CTA TTA TGA GTT CTG CCA AGA AGA TCG G		this study
P60	PciI-cadB-TAA-Rev	CAC ATG TAA TGT GCG TTA GAC GCG GTG T	*Pci*I	this study
P61	NP-CadC-cadA-OL-Rev	GCA ATA ACG TTC ATA ATA GAA ACT CAT TCG AAA AGG GAA TGA TG		this study
P62	NP-CadC-cadA-OL-Fw	GAG TTT CTA TTA TGA ACG TTA TTG CAA TAT TGA ATC ACA TG		this study
P63	BspHI-cadA-TAA-Rev	GCT CAT GAA TTT TTT GCT TTC TTC TTT CAA TAC CTT AAC	*BspH*I	this study
P64	PciI-amiB-rev	CAC ATG TCG TTT GGC AGC GTG CGA TCT GG	*Pci*I	this study
P65	flhC-OL-Fw	GTT TCT ATT ATG AGT GAA AAA AGC ATT GTT CAG GAA GC		this study
P66	flhC-OL-Rev	CTT TTT TCA CTC ATA ATA GAA ACT CAT TCG AAA AGG GAA TGA T		this study
P67	PciI-flhC-rev	CAC ATG TCA ACA GCC TGT ACT CTC TGT TCA	*Pci*I	this study
P68	nlpD-OL-Fw	GTT TCT ATT ATG GGC TGT TCT GAC ACT TCA AAT C		this study

	Name	Sequence	Restric-tion site	Source
P69	nlpD-OL-Rev	GAA CAG CCC ATA ATA GAA ACT CAT TCG AAA AGG GAA T		this study
P70	PciI-nlpD-rev	CAC ATG TCT CGC TGC GGC AAA TAA CGC AGC	*Pci*I	this study
P71	rzoR-OL-Fw	GTT TCT ATT ATG TGC ACA TCA AAG CAG TCT GTC		this study
P72	rzoR-OL-Rev	GAT GTG CAC ATA ATA GAA ACT CAT TCG AAA AGG GAA TGA T		this study
P73	PciI-rzoR-rev	CAC ATG TCC CAG TCG TTC CCG GAG GGT GAA	*Pci*I	this study
P74	tonB-OL-Fw	GTT TCT ATT ATG GTA CAT CAG GTT ATT GAA CTA CCT G		this study
P75	tonB-OL-Rev	GAT GTA CCA TAA TAG AAA CTC ATT CGA AAA GGG AAT GAT		this study
P76	PciI-tonB-rev	CAC ATG TC CTG AAT TTC GGT GGT GCC GTT	*Pci*I	this study
P77	uvrB-OL-Fw	GTT TCT ATT ATG AGT AAA CCG TTC AAA CTG AAT TCC G		this study
P78	uvrB-OL-Rev	GTT TAC TCA TAA TAG AAA CTC ATT CGA AAA GGG AAT GAT G		this study
P79	PciI-uvrB-rev	CAC ATG TCC GAT GCC GCG ATA AAC AGC TCA	*Pci*I	this study
P80	hisD-TF-OL-Fw	GTT TCT ATT ATG AGC TTT AAC ACA ATC ATT GAC TGG AAT AG		this study
P81	hisD-TF-OL-Rev	TGT TAA AGC TCA TAA TAG AAA CTC ATT CGA AAA GGG AAT GAT		this study
P82	PciI-hisD-TF-rev	CAC ATG TCT GCT TGC TCC TTA AGG GCG TTA AC	*Pci*I	this study
P83	flhC-TF-OL-Fw-APP	GCG CGG AAG CGC ACC GCC GAA AG		this study
P84	flhC-TF-OL-Rev-APP	CTT TCG GCG GTG CGC TTC CGC GC		this study
P85	flhC-TF-OL-FW-pap	CTG CGC GGA AGC CCA GCG CCG AAA GGC ATG		this study
P86	flhC-TF-OL-rev-pap	CAT GCC TTT CGG CGC TGG GCT TCC GCG CAG		this study
P87	flhC-TF-OL-FW-aaa	CTG CGC GGA AGC GCA GCG GCG AAA GGC ATG		this study
P88	flhC-TF-OL-rev-aaa	CAT GCC TTT CGC CGC TGC GCT TCC GCG CAG		this study
qRT-PCR-Primer				
P89	Q-PCR-recA-Fw	CGG TTC GCT TTC ACT GGA TAT CG		this study
P90	Q-PCR-recA-Rev	CCT GCA GCG TCA GCG TGG T		this study
P91	Q-PCR-rpoD-Fw	GCT GGC TGA AAA CAC CGC GG		this study
P92	Q-PCR-rpoD-Rev	GCC CAT TTC ACG CAT GTA CAT GC		this study
P93	Q-PCR-cadC-Fw	CGC AGA GTA TCT CAG AAC TAC GTA		this study
P94	Q-PCR-cadC-Rev	CCT CTC CCT CTT CTT CGC TGT		this study
P95	Q-PCR-cadA-Fw	CGC GTT CGC TAA TAC GTA TTC CA		this study
P96	Q-PCR-cadA-Rev	CGT CAG TGG TCT GCT TGA TCT TAT		this study
P97	Q-PCR-cadB-Fw	CGA CTG GCA ACA AAA AAC CCG C		this study
P98	Q-PCR-cadB-Rev	GCC AGG TTA CCA ATC CAG TTA GC		this study
Primer deletion strains				
P99	*yjeK*-mid-50	GAT TGT GAT CCC GGC ACG TAT CAC CGA GGC GCT GGT TGA ATG CTT TGC CCA ATT AAC CCT CAC TAA AGG GCG		(Kraxenberger, 2006)
P100	*yjeK*-down-50	AAG CGT AGC GAA TCA GGC AAT TTT AAT GTT TAA CTT CCC TGT TTA ATC AGT AAT ACG ACT CAC TAT AGG GCT C		(Kraxenberger, 2006)

	Name	Sequence	Restric-tion site	Source
P101	*cadB*-rpsl-up	ATA CAT CAT GAC CCG GAC TCC AAA TTC AAA AAT GAA ATT AGG AGA AGA GCA TGG CAA CAG TTA ACC AGC TGG TAC GC		this study
P102	*cadA*-rpsl-up	TTC ATT TTG TCC CAT GTG TTG GGA GGG GCC TTT TTT ACC TGG AGA TAT GAC TAT GGC AAC AGT TAA CCA GCT GGT ACG C		this study
P103	*cadA*-neo-low	CTT ATG AGC AAA AAA GGG AAG TGG CAA GCC ACT TCC CTT GTA CGA GCT AAT CAG AAG AAC TCG TCA AGA AGG CG		this study
P104	*cadB*-lacZ-up	ATA CAT CAT GAC CCG GAC TCC AAA TTC AAA AAT GAA ATT AGG AGA AGA GCA TGA CCA TGA TTA CGG ATT CAC TG		this study
P105	*cadA*-lacZ-up	TTC ATT TTG TCC CAT GTG TTG GGA GGG GCC TTT TTT ACC TGG AGA TAT GAC TAT GAC CAT GAT TAC GGA TTC ACT G		this study
P106	*cadA*-lacZ-low	CTT ATG AGC AAA AAA GGG AAG TGG CAA GCC ACT TCC CTT GTA CGA GCT AAT TAT TTT TGA CAC CAG ACC AAC TG		this study
P107	cadC neo FRT up	CGT TGG CGA ATG GCT TGT TAC TCC GTC CAT AAA CCA AAT TAG CCG CAA TGA ATT AAC CCT CAC TAA AGG GCG		(Schüppel, 2010)
P108	cadC neo FRT low	TTC AAT CCA GTA AAG GGT GTT TGC CCC TGG GCG TAA ATT AAA GGC GGT GAT AAT ACG ACT CAC TAT AGG GCT C		(Schüppel, 2010)
P109	yjeK_642_cat_fw	GAT TGT GAT CCC GGC ACG TAT CAC CGA GGC GCT GGT TGA ATG CTT TGC CGT GTA GGC TGG AGC TGC TTC		this study
P110	yjeK_642_cat_rev	AAG CGT AGC GAA TCA GGC AAT TTT AAT GTT TAA CTT CCC TGT TTA ATC AGG GAC CAT GGC TAA TTC CCA T		this study
P111	yjeA_rpsl_up	TCG CGT TGC GAG TAG ACT TCG TGC CCT TGT CAA AAA CTG GAG ATT TAA CTG GCC TGG TGA TGA TGG CGG GAT CG		this study
P112	yjeA_neo_low	ATG GCG CTT ATC ACG CCA TTC TTC GCT GTT AAT TCA GTA ATT TTT CAG AAT CAG AAG AAC TCG TCA AGA AGG CG		this study
P113	Hfq_Cm_fw	TCA GAA TCG AAA GGT TCA AAG TAC AAA TAA GCA TAT AAG GAA AAG AGA GAG TGT AGG CTG GAG CTG CTT C		this study
P114	Hfq_Cm_rev	TTG CGC GGA AGT ATT CTG CGC GCT GCT ACC ATG ATG GTA GTT ACT GCT GGA TGG GAA TTA GCC ATG GTC C		this study
P115	Hflx_750_Cm-fw	GGA GCC AGC GAT CCT GCG TTC CCC GCT GAT CTA TTT AGA GGG TTA TAC GCG TGT AGG CTG GAG CTG CTT C		this study
P116	Hflx_750_Cm-rev	AAA TGC CGC CAC CAG ATC GTG7CGG CAG GTG GCG AAT AAA CCC TAC GGT ATA TGG GAA TTA GCC ATG GTC C		this study
P117	His-C-terminal-Hfq_sense	CCG AAT TCA TGG CTA AGG GGC AAT CTT TA	*Eco*RI	this study
P118	His-C-terminal-Hfq_antisense	AAC ATA TGT TCG GTT TCT TCG CTG TCC TG	*Nde*I	this study

	Name	Sequence	Restric-tion site	Source
P119	His-N-terminal-YjeA_sense	AAC ATA TGA GCG AAA CGG CAT CCT GGC A	*Nde*I	this study
P120	His-N-terminal-YjeA_antisense	CCT CTA GAT TAT GCC CGG TCA ACG CTA AAG	*Xba*I	this study
Check Primer deletion strains				
P121	defp-chk-Fw	GCA AAG CAT TCA ACC AGC GCC		this study
P122	defp-chk-Rev	GAC TAA AGC CGT GGG TAT ATT TCA G		this study
P123	*cadB*-fw-seq	CAG TTT GGT CTG GTC AGG AAA TAG		this study
P124	*cadA*-rev-seq	GTG AGG GTG TTT TCA TGT GTT CTC		this study
P125	cadC up	GCA ACA ACC TGT AGT TCG		this study
P126	cadC low	AAC AGA AGT CTG GAA TAT ACC		this study
P127	*yjeA*-fw-seq	GCC CTT GTC AAA AAC TGG AG		this study
P128	*yjeA*-rev-seq	CGC TGT TAA TTC AGT AAT TTT TCA G		this study
P129	walk-for-2	GCC GTA CTC ACT CTA TAT AAA CAG AA		(Kraxenberger, 2006)
P130	walk-on-reverse	ATC GCG CCC GGA TTC TCC ACC GAC		(Kraxenberger, 2006)
P131	Hflx_check_fw	GTT TCG GGC TGT TTT TTT ACA CG		this study
P132	Hflx_check_rev	GTT ATG CGG TGA TCC CTG TTG		this study
P133	Hns-Cm-fw	ATG AGC GAA GCA CTT AAA ATT CTG AAC AAC ATC CGT ACT CTT CGT GCG CAG TGT AGG CTG GAG CTG CTT C		this study
P134	Hns-Cm-rev	TTA TTG CTT GAT CAG GAA ATC GTC GAG GGA TTT ACC TTG CTC ATC CAT TGA TGG GAA TTA GCC ATG GTC C		this study
P135	dhns-chk-Fw	GGT AGT GGG TGA TGA ACC GGT AG		this study
P136	dhns-chk-Rev	GTG GGT ATA TGC GTT CTC CCT TAC		this study
Sequencing Primers				
P137	T7 Promoter	TAA TAC GAC TCA CTA TAG GG		
P138	T7 Terminator	TAT GCT AGT TAT TGC TCA G		
P139	Seq33-Fw-100	GGC GTC CAC ACT TTG CTA TGC		this study
P140	pBAD-HisA-rev	CAG TTC CCT ACT CTC GCA TG		this study
P141	M13 (-21)uni	TGT AAA ACG ACG GCC AGT		
P142	M13 (-29)rev	CAG GAA ACA GCT ATG ACC		
P143	Hfq_antisense	CCT CTA GAT TAT TCG GTT TCT TCG CTG TC		this study
Artificial introduction of selected aa at positon two of the CadC ORF				
P144	PciI-CadC-TF02-Rev (His)	GCA CAT GTG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P145	PciI-CadC-TF02-Gln-Rev	GCA CAT GTG TTG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P146	PciI-CadC-TF02-Gly-GGC-Rev	GCA CAT GTG GCC CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P147	PciI-CadC-TF02-Ala-Rev (GCG)	GCA CAT GTG CGC CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P148	PciI-CadC-TF02-Leu-Rev	GCA CAT GTG CAG CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P149	PciI-CadC-TF02-Met-Rev	GCA CAT GTG CAT CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P150	PciI-CadC-TF02-Phe-TTT-Rev	GCA CAT GTG AAA CAT AAT AGA AAC TCA TTC GAA AAG GG	*Pci*I	this study
P151	CadC-Gly-Fw	GAG TTT CTA TTA TGG GCC AAC AAC CTG TAG		this study
P152	CadC-Gly-Rev	CTA CAG GTT GTT GGC CCA TAA TAG AAA CTC		this study

	Name	Sequence	Restric-tion site	Source
P153	CadC-Phe-Fw	GAG TTT CTA TTA TGT TCC AAC AAC CTG TAG		this study
P154	CadC-Phe-Rev	CTA CAG GTT GTT GGA ACA TAA TAG AAA CTC		this study
Primer for in vitro studies				
P155	EF-P K34 FOR	GAATTCGTAAAACCGGGTGCA GGCCAGGCATTTGCTCGC		Dr. A. Starosta
P156	EF-P K34 REV	GCGAGCAAATGCCTGGCCTGCACCCG GTTTTACGAATTC		Dr. A. Starosta
P157	Flk_{1-10}-REV	CTA GTT ATT GCT CAG CGG TTT AAG GAG GAG GGC CGG AAA TAG GTT G		Dr. A. Starosta
P158	$FlhC_{1-53}$-REV	CTA GTT ATT GCT CAG CGG TTT ACG GCG GTG GGC TTC CGC GCA GTT C		Dr. A. Starosta
P159	$RzoR_{1-18}$-REV	CTA GTT ATT GCT CAG CGG TTT ACG GAG GCG GTG GTG GCT TCA C		Dr. A. Starosta
P160	$CadC_{1-125}$-REV	CTA GTT ATT GCT CAG CGG TTT ATG GTA TAG GGG GAG GCG AAG ATA G		Dr. A. Starosta
P161	CadC-NdeI-sense	AGG AAA GGA CAT ATG CAA CAA CCT GTA GTT CG	*Nde*I	(Küper & Jung, 2005)
P162	CadC-BamHI-stop	AGT GGA TCC TTA TTC TGA AGC AAG AAA TTT GTC C	*BamH*I	(Küper & Jung, 2005)
P163	BamHI-TAA-CadC-131-Rev	GCC GGA TCC TTA TTATTA TGG TAT AGG GGG AGG CGA AGA TA	*BamH*I	this study
Bacterial two hybrid studies				
P164	XbaI-BTH-EF-P-Fw	CGT CTA GAG GCA ACG TAC TAT AGC AAC GAT TTT C	*Xba*I	(Krönauer, 2011)
P165	BamHI-BTH-EF-P-Rev	CTG GAT CCG CCT TCA CGC GAG AGA CGT ATT CAC	*BamH*I	(Krönauer, 2011)
P166	XbaI-BTH-CadC-Fw	AGT CTA GAG CAA CAA CCT GTA GTT CGC GTT G	*Xba*I	(Krönauer, 2011)
P167	BamHI-BTH-CadC-Rev	CTG GAT CCG CTT CTG AAG CAA GAA ATT TGT CGA GAT A	*BamH*I	(Krönauer, 2011)
P168	XbaI-BTH-LysP-Fw	AGT CTA GAG GTT TCC GAA ACT AAA ACC ACA GAA G	*Xba*I	(Krönauer, 2011)
P169	BamHI-BTH-LysP-Rev	CTG GAT CCG CTT TCT TAT CGT TCT GCG GGA ACT TC	*BamH*I	(Krönauer, 2011)
P170	XbaI-BTH-YjeA-Fw	AGT CTA GAG AGC GAA ACG GCA TCC TGG	*Xba*I	(Krönauer, 2011)
P171	BamHI-BTH-YjeA-Rev	CTG GAT CCG CTG CCC GGT CAA CGC TAA AGG	*BamH*I	(Krönauer, 2011)
P172	BamHI-BTH-H-NS-Rev	CGT CTA GAG AGC GAA GCA CTT AAA ATT CTG AAC A	*Xba*I	(Krönauer, 2011)
P173	XbaI-BTH-H-NS-Fw	CTG GAT CCG CTT GCT TGA TCA GGA AAT CGT CGA G	*BamH*I	(Krönauer, 2011)
P174	XbaI-BTH-YjeK-Fw	AGT CTA GAG GCG CAT ATT GTA ACC CTA AAT ACC	*Xba*I	(Krönauer, 2011)
P175	BamHI-BTH-YjeK-Rev	CTG GAT CCG CCT GCT GGC GTA GCT GGA GAT	*BamH*I	(Krönauer, 2011)
Generation of CadC Ly to Ala mutants				
P176	CadC_K59A_antisense	CAA TAC TTC TCG CCC AGA CAT TAT C		this study
P177	CadC_K59A_sense	GAT AAT GTC TGG GCG AGA AGT ATT G		this study
P178	CadC_K77A_antisense	CTT TTA ATG ACG CAC GTA GTT C		this study
P179	CadC_K77A_sense	GAA CTA CGT GCG TCA TTA AAA G		this study
P180	CadC_K80A_antisense	CAT CAT TAT CTG CTA ATG ACT TAC G		this study
P181	CadC_K80A_sense	CGT AAG TCA TTA GCA GAT AAT GAT G		this study
P182	CadC_K95A_antisense	TAT AGC CGC GCG CTG GTA CAG		this study

	Name	Sequence	Restric-tion site	Source
P183	CadC_K95A_sense	CTG TAC CAG CGC GCG GCT ATA		this study
P184	CadC_K99A_antisense	CAC CAT TAA TGC ATA GCC GC		this study
P185	CadC_K99A_sense	GCG GCT ATG CAT TAA TGG TG		this study
P186	CadC_K80A_antisense	GAA TCG TTT GCT TGC AAC TGG		this study
P187	CadC_K80A_sense	CCA GTT GCA AGC AAA CGA TTC		this study
Generation of CadC Ly to Arg mutants				
P188	CadC_K59R_antisense	CAATACTTCTCCTCCAGACATTATC		this study
P189	CadC_K59R_sense	GATAATGTCTGGAGGAGAAGTATTG		this study
P190	CadC_K77R_antisense	ATC TTT TAA TGA CCT ACG TAG TTC		this study
P191	CadC_K77R_sense	GAA CTA CGT AGG TCA TTA AAA GAT		this study
P192	CadC_K80R_antisense	CAT CAT TAT CTG CTA ATG ACT TAC G		this study
P193	CadC_K80R_sense	CGT AAG TCA TTA GCA GAT AAT GAT G		this study
P194	CadC_K95R_antisense	TAT AGC CGC GCC TTG GTA CAG		this study
P195	CadC_K95R_sense	CTG TAC CAA GGC GCG GCT ATA		this study
P196	CadC_K99R_antisense	CAC CAT TAA TCT ATA GCC GCG		this study
P197	CadC_K99R_sense	CGC GGC TAT AGA TTA ATG GTG		this study
P198	CadC_K80R_antisense	CAT CAT TAT CTC TTA ATG ACT TAC G		this study
P199	CadC_K80R_sense	CGT AAG TCA TTA AGA GAT AAT GAT G		this study
P_{cadA}-*lacZ* fusions				
P200	XbaI-PcadA79_fw	AAT CTA GAT TAA AAG TAT TTT CCG AGG CTC CTC	*Xba*I	this study
P201	XbaI-PcadA100_fw	AAT CTA GAC TTC ATC GTC AGC CTG ATT ATC CT	*Xba*I	this study
P202	XbaI-PcadA200_fw	AAT CTA GAG TTA ACA TCC GCA ACT TTG TCA GC	*Xba*I	this study
P203	XbaI-PcadA300_fw	AAT CTA GAG TAA AGC ATC TGA CCT GTT CGG TG	*Xba*I	this study
P204	XbaI-PcadA400_fw	AAT CTA GAG AAG TCG ACA GCA ACG GTA TTC C	*Xba*I	this study
P205	XbaI-PcadA500_fw	AAT CTA GAC TTT GCG TGC CTG ACT TCT CTG	*Xba*I	this study
P206	XbaI-PcadA600_fw	AAT CTA GAC TAT CCT CGG TAA CTG GGC TGC GC	*Xba*I	this study
P207	XbaI-PcadA700_fw	AAT CTA GAG TTA AAA ACC CGA AAC GTA CCG TTC	*Xba*I	this study
P208	XbaI-PcadA800_fw	AAT CTA GAG AAT ACT GCG GAT ACC ACT GAT GG	*Xba*I	this study
P209	PciI-PcadA600_rev	AAA CAT GTA GTC ATA TCT CCA GGT AAA AAA GGC CCC TC	*Pci*I	this study

2.3 Cultivation techniques

According to the NaCl modification of Miller (Miller, 1972), Lysogeny Broth (LB) was used as complex medium (Bertani, 1951). When indicated, LB was buffered with 100 mM sodium-phosphate to pH 7.6 or 5.8. Potassium buffered minimal medium (KE, pH 7.6 or 5.8) (Epstein & Kim, 1971) was supplemented with 0.4% (w/v) glucose as carbon source and, when indicated, with 10 mM lysine. LB plates contained 1.5% (w/v) agar. Antibiotics were used when necessary with following concentrations: 100 µg/ml ampicillin sodium salt, 50 µg/ml kanamycin sulfate, 34 µg/ml chloramphenicol, 50 µg/ml streptomycin sulfate, 15 µg/ml tetracycline hydrochloride or 15 µg/ml gentamycin sulfate. For blue-white selection, LB agar plates were additionally supplemented with 80 µM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Unless otherwise stated, cells were grown at 37 °C. Under aerobic conditions, cells were shaken in baffled flasks (up to 1 l) or test tubes (up to 5 ml) at 200 rpm. Overnight cultures were always incubated aerobically. Microaerobic conditions were achieved by growing cells in bottleneck-filled flasks or Greiner tubes with minimal agitation (2 rpm). To control cell growth, the optical densitiy was determined at 600 nm (OD_{600}) in an Ultrospec 2100 pro photometer (Amersham Biosciences).

2.3.1 Purification of YjeK

For overproduction of YjeK, an overnight culture of strain Bl21(DE3) harboring plasmid pET16b-His_6-YjeK was diluted (1:100) and aerobically grown in LB medium, which was supplemented with ampicillin. When cells had reached an OD_{600} of ≈ 0.5, 0.5 mM IPTG was added. After shaking for 3 h, cells were harvested by centrifugation (10 min; 6000 rpm; 4 °C; Sorvall® Evolution™ RC) and washed once with a low ionic washing buffer containing 50 mM Tris/HCl (pH 8.0) and 10 mM $MgCl_2$. The cell pellet was shock-frozen and stored at -80 °C. YjeK was then purified using Ni^{2+}-NTA column chromatography (section 2.5.1).

2.3.2 Lysine decarboxylase and β-galactosidase activity tests

Cultivations for the lysine decarboxylase (LDC, CadA) and β-galactosidase activity tests were done in KE minimal medium or LB medium equally. To this end, an overnight culture was diluted to an OD_{600} of 0.05 in the corresponding medium. To induce the *cad* operon, 10 ml

cells were incubated microaerobically in low pH buffered medium and additionally with 10 mM lysine in KE minimal medium. When cells had reached an OD_{600} of 0.3 - 0.7, 1 ml was harvested in a 2 ml Eppendorf tube by centrifugation (10 min; 13,200 rpm; 4 °C; Eppendorf 5415R). Before measuring the CadA or β-galactosidase activity (section 2.5.6 or 2.5.7), cell pellets could be stored at -20 °C.

For the bacterial two hybrid assay (section 2.5.8), cells were incubated at 30 °C in 4 ml LB medium, which was supplemented with ampicillin, kanamycin and IPTG. At an OD_{600} of 0.5 - 0.9, cells were harvested by centrifugation (5 min; 13,200 rpm; 4 °C) and stored at -20°C.

2.3.3 Cultivations for RNA isolation

For Northern Blot analysis (section 2.4.12), wildtype cells or Δ*yjeK* cells were first grown aerobically in 5 l KE minimal medium (pH 7.6), until they reached an OD_{600} of 0.6 - 0.8. Then, cells were harvested by centrifugation (5 min, 6000 rpm, 37 °C) and immediately shifted to a 5 l fermenter containing KE minimal medium pH 5.8 with 10 mM lysine. To avoid sedimentations, the medium was stirred at 50 rpm. The pH was monitored by a pH sensor. At time points 0, 1, 5, 10, and 20 min after the shift, 10 ml were harvested in Greiner tubes by centrifugation (10 min; 10,000 rpm; 4 °C). Cell pellets were shock-frozen and stored at -80 °C.

For quantitative real time PCR (qRT-PCR, section 2.4.13), cells grown exponentially to an OD_{600} of 0.4 - 0.6 in pH 7.6 buffered KE minimal medium were harvested by centrifugation (10 min; 4000 rpm; 4 °C). Subsequently, cells were exposed either to non-inducing conditions (pH 7.6) or inducing conditions (pH 5.8 + 10 mM lysine) for 25 min (Küper & Jung, 2005). At time points 0 min and 25 min, 10 ml of the culture were harvested by centrifugation (20 min, 4000 rpm, 0 °C). Cell pellets were immediately frozen in liquid nitrogen and stored at -80°C for further experiments.

Pellets were further used for RNA isolation (section 2.4.10).

2.3.4 CadC membrane vesicles

For quantification of CadC in membrane vesicles (section 2.5.2), strains were aerobically cultivated in 1 l buffered KE or LB medium (pH 7.6). Cells having an OD_{600} of ≈ 1 were harvested by centrifugation (5 min, 6000 rpm, 37 °C) and shifted to inducing conditions (KE medium: pH 5.8 with 10 mM lysine, LB medium: pH 5.8) for 1 h without shaking. Cells grown in LB medium were additionally washed once with washing buffer containing 50 mM

Tris/HCl (pH 8.0) and 10 mM $MgCl_2$, before harvesting them by centrifugation. Cell pellets were shock-frozen and stored at -80°C.

2.3.5 Culture storage

For storage, a fresh overnight culture was mixed with 17% (v/v) glycerol and immediately shock-frozen. Afterwards, the strain was stored at -20°C or -80°C.

2.4 Molecular biological and genetic methods

2.4.1 Isolation of plasmids and genomic DNA

Plasmid isolations were done according to the manufacturer´s directions of the HiYield plasmid mini-kit (Süd Laborbedarf). Plasmids were stored at -20°C or -80°C.
Genomic DNA was isolated according to the manufacturer´s directions of the DNeasy 96 blood and tissue kit (Quiagen). Isolated DNA was diluted in 200 µl ddH_2O and stored at -20°C.

2.4.2 Modification of DNA

The standard DNA techniques were, unless otherwise stated, carried out according to „Molecular Cloning, a Laboratory Handbook“ (Sambrook & Russel, 2001). In vitro modifications of DNA molecules, like ligations and restrictions, were performed as recommended by the manufacturer´s manual. The ends of linearized vectors were dephosphorylated by the enzyme alkaline phosphatase to avoid religations.

2.4.3 Polymerase chain reaction

Taq and Phusion DNA polymerases were employed for DNA amplification according to the manufacturer´s directions. As templates, 20 - 100 ng genomic DNA were used. To test the insertion of a fragment into the bacterial genome after transformation, a colony PCR was done by transferring cells of the clone of interest to the PCR tube with a sterile toothpick. The PCR was performed according to Mullis *et al.* (Mullis *et al.*, 1986). Cyclers were products from Eppendorf and Biorad.

2.4.4 Electrophoretic separation of DNA

DNA fragments were separated by agarose gel electrophoresis. Before loading the DNA on the gel, 10 x DNA loading dye [50% glycerol (v/v); 0.1 M EDTA; 1% SDS (w/v); 0.1% bromphenol blue (w/v)] was added. Gels consisted of 1% - 2% (w/v) agarose in 1 x TAE buffer (40 mM acetic acid; 1 mM EDTA; 40 mM Tris) and 0.2 µg/ml ethidium bromide. As standard DNA marker, the 2-log DNA ladder from NEB (0.1 - 10 kb) was employed. The gel run was carried out at 100 V in a „Mini Sub DNA Cell“ - and a “Wide Mini Sub cell GT”-agarose gel chamber (Biorad, München), respectively. DNA was detected by an UV-transilluminator (304 nm) and gels were documented by the “Cybertec”-gel documentation system of Peqlab.

2.4.5 DNA gel extraction and determination of nucleic acid concentration

DNA fragments were purified from agarose gels by using the HiYield DNA extraction kit (Süd-Laborbedarf) according to the manufacturer´s instructions. The concentrations of DNA and RNA were measured by using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.4.6 DNA sequencing analysis

Sequencing analysis was performed by the sequencing service of the Institute of Genetics of the Ludwig-Maximilians-University (LMU) using the “ABI Prism Model 3730” (Applied Biosystems). The sequencing principle was based on the “Cycle Sequencing” of Sanger *et al.* (Sanger *et al.*, 1977). Used primers are listed in Table 4.

2.4.7 Strain construction

Strains MG-CL-1, MG-CR, MG-CL-12-*hflx*, MG-CL-12-*yjeA* and SU2 were constructed by using the pRED®/ET® recombination technology together with the *rpsL* counter-selection (Heermann *et al.*, 2008) in accordance to the technical protocol of the Quick & Easy *E. coli* Gene Deletion Kit of Gene Bridges (www.genebridges.com). For the construction of strains SU1, SU3, SU4 and MG-CL-12-*hfq*, plasmid pKD3 was employed for fragment amplification, in which each end of the pKD3 chloramphenicol cassette was enlarged by 50 base pairs flanking the region of $yjeK_{643\text{-}1029}$, $hns_{1\text{-}230}$ or $hfq_{1\text{-}230}$. Gene deletion was then

performed using the same protocol as mentioned above. Strain MG-CL-12-*yjeK* was constructed by P1 phage transduction (Miller, 1972). Preparation of the phage lysates of donor strains TK1 and transduction to the recipient strains was performed as described previously (Leder *et al.*, 1977).

2.4.8 Plasmid construction

Plasmids and their constructions are described in Table 3. Some of the plasmids, which have been recently published in Ude *et al.* (2013), were constructed in cooperation with Dr. Jürgen Lassak.

2.4.9 Preparation of competent cells and transformation

For plasmid transformation, chemically competent *E. coli* cells were prepared using a modified RbCl procedure (Promega, 1994). To this end, 10 ml fresh LB medium supplemented with the corresponding antibiotic were inoculated with 100 µl overnight culture. Cells exponentially grown to an OD_{600} of 0.3 - 0.6 were harvested by centrifugation (5 min, 5000 rpm, 4 °C, Eppendorf table centrifuge). The pellet was resuspended in 5 ml cold solution A (10 mM MOPS; 10 mM RbCl; pH 7.0), and cells were again centrifuged. After resuspending the pellet in 5 ml cold solution B (100 mM MOPS; 50 mM $CaCl_2$; 10 mM RbCl; pH 6.5), cells were incubated 30 min on ice and centrifuged. The supernatant was discarded and cells were dissolved in 1 ml cold solution B. After 1 h incubation on ice, 200 µl competent cells were mixed with 1 µl plasmid or an entire ligation sample. The mixture was left on ice for another hour. Subsequently, cells were exposed to heat shock (max. 90 sec, 42 °C) and shaken at 37 °C and 11,000 rpm for 1 h. Afterwards, 100 µl (for plasmid transformation) or the whole sample (for ligation transformation) was plated out on a selective agar plate and incubated overnight at 37 °C.

For the transformation of linear DNA, electrocompetent cells were prepared according to the technical protocol of the Quick & Easy *E. coli* Gene Deletion Kit of Gene Bridges (www.genebridges.com). 40 µl of the competent cells were mixed with 0.4 µg - 1 µg DNA and filled into an ice cold electroporation cuvette. Cells were immediately electroporated by an electric pulse of 2.5 kV on average, using the MicroPulser electroporator of Biorad.

2.4.10 Isolation of RNA

Total RNA was extracted from *E. coli* cells using the hot-phenol method (Aiba *et al.*, 1981). All buffers were prepared using 0.1% (v/v) DEPC water. Cell pellets were resuspended in 200 µl cold Tris/HCl buffer (20 mM Tris/HCl, pH 8.0) and 3 ml lysis buffer A [20 mM sodium acetate; 0.5% (w/v) SDS; 1 mM EDTA, pH 8.0; adjusted to pH 5.5 with acetic acid]. The RNA was extracted by adding 3 ml hot phenol (60°C) and incubating the sampls at 60 °C for 5 min. After centrifugation (10 min; 13,000 rpm; 4 °C), the aqueous upper layer was transferred in a new tube. Subsequently, 3 ml hot phenol/chloroform/isoamylalcohol (25:24:1) were added and the samples were again centrifuged. The upper layer was decanted in a new Greiner tube. 8 ml cold 100% ethanol was added, and the RNA was precipitated overnight at -20°C. After centrifugation (15 min; 13,000 rpm; 4 °C), the supernatant was discarded and the pellets were washed with 70% (v/v) cold ethanol. Subsequently, the pellets were dried at room temperature for approx. 30 min and resuspended in 0.1% (v/v) DEPC water. Residual chromosomal DNA was removed by DNAse I digestion (Thermo Scientific / Fermentas) according to the manufacturer´s instruction. The purified RNA was then used for Northern Blot analysis (section 2.4.12) and qRT-PCR (section 2.4.13) and stored at -20°C.
RNA isolation for qRT-PCR experiments were done in cooperation with Dr. Jürgen Lassak.

2.4.11 Denaturated gel electrophoresis

Before the Northern Blot, 1.2% (w/v) denaturated agarose gels were prepared to avoid secondary structures of the RNA according to a standard protocol (Sambrook & Russel, 2001). 120 ml of the gel contained 12 ml 10 x MOPS buffer (80 mM sodium acetat; 10 mM EDTA; 200 mM MOPS; pH 7.0) and 3.6 ml formaldehyde (37%). 20 µg of total RNA was mixed with 1 volume of pre-mix solution [64.5% (v/v) formamide; 8.4% (v/v) formaldehyde; 1.3 x MOPS buffer], 0.002 ng/µl ethidium bromide and 1/10 volume 10 x RNA dye [50% (w/v) glycerol; 0.8% (w/v) bromphenol blue; 0.8% (w/v) xylencyanol; 1 mM EDTA, pH 8]. Probes were heated for 10 min at 65 °C and put on ice. Afterwards, probes and the RNA ladder (NEB) were loaded on the gel, which ran 2 - 3 h at 60 V. The loading buffer was composed of 1 x MOPS. All solutions were prepared using 0.1% (v/v) DEPC water.

2.4.12 Northern Blot analysis

The transfer of RNA from the denaturated agarose gel to the Hybond-N nylon membrane (GE healthcare) was performed by following a standard protocol (Sambrook & Russel, 2001). 20 x SSC (3 M NaCl; 0.3 M sodium citrate; pH 7.0) was used as transfer buffer. The RNA was fixed on the membrane by UV light (304 nm), and the membrane was dried for 4 h at 80 °C. After the pre-hybridization of the membrane in 10 ml hybridization buffer [5 x SSC (0.75 M NaCl; 75 mM sodium citrate; pH 7.0); 5 x Denhardt´s reagents (Denhardt, 1966), 100 µg/ml denaturated salmon sperm DNA; 0.1% (w/v) SDS] at 60 °C for 1 h, the radioactive probe (against *cadA*, *cadB* or *cadC*) was prepared according to the "Rediprime II DNA Labeling System" kit (GE Healthcare). The labeling was done by using 0.05 mCi (millicurie) of [α^{32}-P]-dCTP per sample. The probe was mixed with 7.5 ml hybridization buffer and the membrane was incubated with the mixture at 60 °C overnight. Afterwards, the membrane was washed three times with 60 ml washing solution [2x SSC (0.3 M NaCl; 30 mM sodium citrate; pH 7.0)] for 5 min at 42 °C. The membrane was dried on air, and the exposition was done overnight by using a "Storage Phosphor Screen" (GE healthcare). Afterwards, the blotted screen was used for signal detection by a phosphoimager (Typhoon Trio, GE healthcare).

2.4.13 qRT-PCR

Extracted total RNA was applied as template for random-primed first-strand cDNA synthesis by using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific / Fermentas) according to the manufacturer´s instructions. The cDNA was then used as template for qRT-PCR (IQ5 optical system; Bio-Rad) by using the IQ SYBR Green Supermix (Bio-Rad). Samples were assayed at least in duplicates. The signal was normalized to *recA* (Gralnick *et al.*, 2005). The cycle threshold (C_t) was automatically determined by use of the IQ5 optical system software (Bio-Rad) after 40 cycles. The efficiency of each primer pair was determined using five different concentrations of *E. coli* chromosomal DNA (100 µg/ml, 10 µg/ml, 1 µg/ml, 100 ng/ml, 10 ng/ml) as templates for qRT-PCR.
qRT-PCR experiments were done in cooperation with Dr. Jürgen Lassak.

2.5 Biochemical and analytical methods

2.5.1 Purification of YjeK and CadC

5´-His_6-tagged YjeK was purified by Ni^{2+}-NTA affinity chromatography using protocols 9 and 12 of the manual "The QIAexpressionist" from Qiagen (http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAexpressionist_EN.pdf). Following modifications were done: The lysis buffer was supplemented with 0.5 mM PMSF (phenylmethanesulfonylfluoride). The loaded column was washed twice with 10 ml ddH_2O, and then 4 times with washing buffer. The protein was eluated with 12 x 300 µl steps. CadC was produced and purified as described earlier (Küper & Jung, 2005).

2.5.2 Preparation of CadC membrane vesicles

The protocol for preparation of membrane vesicles was based on the work of Siebers & Altendorf (Siebers & Altendorf, 1988), with modification in accordance to Jung *et al.* (Jung *et al.*, 1997). 0.2 g of the cell pellet was resuspended in lysis buffer [50 mM Tris/HCl, pH 7.5; 10% (v/v) glycerol; 0.5 mM PMSF; 10 mM $MgCl_2$; 1 mM DTT; 30 ng/ml DNAse A] and incubated on ice with 10 µg/ml lysozyme for 30 min. Cells were disrupted by sonification (Sonifier W-250D, Branson) and harvested by centrifugation (10 min; 16,000 rpm; 4 °C). The cell debris was discarded and the supernatant was ultracentrifuged (1 h; 45,000 rpm; 4 °C). The pellet was washed (1 mM Tris/HCl, pH 7.5; 3 mM EDTA, pH 8.0; 0.5 mM PMSF) and ultracentifuged for a second time. Afterwards, the pellet was resuspended in TG buffer [50 mM Tris/HCl, pH 7.5; 10% (v/v) glycerol], shock-frozen and stored at -80 °C.

2.5.3 Determination of the protein concentration

The protein concentration in the cytoplasm was determined using the Bradford method as described before (Bradford, 1976). Concentration of proteins in membrane vesicles was determined in accordance to Lowry *et al.* (Lowry *et al.*, 1951) with modifications (Peterson, 1977).

2.5.4 SDS Page

Proteins were separated by denaturating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in accordance to Lämmli (Lämmli, 1970). The stacking gels contained 4.9% (w/v) acrylamide, the separating gels 12.5% (w/v) acrylamide. Before loading on a gel, protein samples were mixed with SDS loading buffer [25 mM Tris/HCl, pH 6.8; 2% (v/v) glycerol; 2% (w/v) SDS; 5% (v/v) β-mercaptoethanol; 0.005% (w/v) bromphenol blue]. As markers, the Unstained Protein Molecular Weight Marker (14 - 116 kDa; Fermentas) and the PageRuler Prestained Protein Marker (10 - 170 kDa; Fermentas) were employed. For the preparation of native gels, the same protocol was employed as mentioned above, but without any SDS, β-mercaptoethanol or DTT. The gels run in "PerfectBlue double gel system Twin S or Twin M" (Biorad) at 200 V in Lämmli buffer [25 mM Tris; 192 mM glycin; 0.1% (w/v) SDS; pH 8.2 - 8.3). Afterwards, gels were stained with Serva Blue G-250 (Coomassie Blue) accordingly to Weber and Osborn (Weber & Osborn, 1969). The decoloration buffer contained 5% (v/v) methanol and 7.5% (v/v) acetic acid.

2.5.5 Western Blot analysis

Proteins, which were first separated by SDS-PAGE, were blotted on a nitrocellulose membrane with 0.45 µm pore diameter (Whatman, Protran BA85) by using a wet-blot apparatus (Mini Trans-Blot Cell, Biorad). The horizontal electrophoresis was performed for 90 min at 300 mA for a small gel (10 x 10 cm) and 90 min at 800 mA for a big gel (14 x 16 cm). The Western Blot analysis was performed in accordance to protocol 8 of the "QIAexpress Detection and Assay Handbook 10/2002" from Qiagen (http://www.qiagen.com/selectlocation.aspx?redirect=%2fliterature%2frender.aspx%3fid%3d129). To detect proteins with histidine (His) - tags, the antibody "Penta-His-Anti-Mouse-IgG" was used as primary antibody with an end concentration of 1:2000, and the "anti-Mouse IgG (chicken) antibody conjugated with alkaline phosphatase (AP)" was employed as secondary antibody (1:5000). To detect CadC, the blot membrane was incubated first with a specific pre-adsorbed CadC antibody (rabbit, 2006) (1:10,000) and then with an "anti-Rabbit IgG antibody, AP conjugated" (1:2500). As last step, the immunoblot was incubated with a dye solution [50 mM sodium carbonate (pH 9.5); 0.01% (w/v) NBT; 0.045% (w/v) BCIP] for 5 - 15 min.

2.5.6 Lysine decarboxylase activity test

Specific lysine decarboxylase (CadA) activity was measured in cell-free extracts as previously described (Lemonnier & Lane, 1998) with minor modifications (Tetsch *et al.*, 2008). 1 ml cells were harvested by centrifugation, and the cell pellet was adjusted to an OD_{600} of 1 with cold potassium phosphate buffer (20 mM, pH 5.6). For cell lysis, 200 µl of the cell suspension were transferred into a new Eppendorf tube, and 20 µl chloroform were added. After vortexing, 10 µl of the upper phase was transferred into 120 µl of pre-warmed reaction buffer [16 mM potassium phosphate buffer, pH 5.6; 0.1 mM pyridoxal phosphate (PLP)]. For each sample, four tubes with reaction buffer were used, whereby two tubes were additionally supplemented with 10 mM lysine as substrate for CadA, whereas the samples without lysine served as negative controls. After 15 min incubation time at 37 °C, 120 µl Na_2CO_3 were added to neutralize the medium and therefore to stop the CadA activity. The lysate was put on ice, and 120 µl trinitrobenzenesulfonic acid (TNBS, 10 mM) were added. TNBS reacts with lysine, but also with the endproduct of CadA, cadaverine, resulting in N,N'-bistrinitrophenyllysine (TNP-lysine) and N,N'-bistrinitrophenylcadaverine (TNP-cadaverine). After mixing the lysate with 1 ml toluol for 20 sec, TNP-cadaverine could be separated from TNP-lysine, as it is soluble in the organic phase of toluol - in contrast to TNP-lysine. The TNP-cadaverine standard curve was done before by Christiane Koller (Koller, 2008). An absorption of 1 at 340 nm corresponds to 106 nmol cadaverine.

2.5.7 β-Galactosidase activity test

β-Galactosidase activities were determined as described before (Tetsch *et al.*, 2008) for at least three independent experiments and are given in Miller units (MU) (Miller, 1992). The significance of the results was determined by applying two-sided students t-test and stating a result as significantly different if $p < 0.05$. For the test, the cell pellet was resuspended in 1 ml buffer Z (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 50 mM β-mercaptoethanol; pH 7.0). After cell lysis with 100 µl chloroform and 50 µl 0.1% (v/v) SDS, lysates were incubated at 30 °C for 5 min. Then, 200 µl ONPG (4 mg/ml) were added as substrate for the β-galactosidase. When yellowing, the reaction was stopped after 0.5 – 10 min with 500 µl sodium carbonate (1 M). Cells were centrifuged (5 min; 13,200 rpm; RT) and the colour was determined photometrically at an absorption of 420 nm using following formula:

$$\beta\text{-galactosidase-activity [Miller-Units]} = (A_{420} * 1000) * (t[\text{min}] * V[\text{ml}] * OD_{600})^{-1}$$

β-Galactosidase activity assays concerning the bacterial two hybrid system were performed by Christina Krönauer in her research internship in the AG K. Jung. β-Galactosidase activity assays concerning the search for the EF-P signal sequence were done in cooperations with Dr. Jürgen Lassak.

2.5.8 Bacterial two hybrid system

This system is based on the split of *Bordetella pertussis* adenylate cyclase into a T18 and a T25 fragment (Karimova *et al.*, 1998; Karimova *et al.*, 2000). Separate fragments reveal no enzymatic activity, but if two proteins come in close proximity, a fully functional adenylate cyclase reconstitutes and allows expression of *lacZ* from a CRP inducible promoter. The resulting β-galactosidase activity can be measured by cleavage of ONPG and reflects the strength of protein-protein interaction. To this end, *cadC*, *lysP*, *hns*, *yjeA*, *yjeK*,and *efp* were cloned into pKT25, pKNT25 and pUT18C (Table 5). The strain *E. coli* BTH101 was then co-transformed with the resulting plasmids leading to strains harboring N/C-terminal T25 fusion in combination with a N-terminal T18 fusion. The β-galactosidase activity was measured as described above.
Bacterial two hybrid system experiments were done in cooperation with Christina Krönauer and Dr. Jürgen Lassak.

2.5.9 In vitro translation assay

In vitro translations of CadC and the proline rich proteins were performed using PURExpress Δaa ΔtRNA (NEB), according to manufacturer's instructions. Briefly, translation was coupled to transcription using PCR templates containing a T7 promoter and supplemented with [^{35}S]-methionine (Perkin Elmer) and 1 µM EF-P. Reactions were incubated at 30°C, 2.5 µl aliquots were removed at specified time points and stopped by placing them on ice or by addition of 20 µM thiostrepton (Sigma). RNase A treatment involved addition of 12.5 µg RNaseA (Roche) per reaction and incubation for 10 min at 30°C. Translation products were separated by double-layered 15%/18% Schägger-von Jagow SDS-PAGE (Schagger & von Jagow, 1987) and detected by autoradiography using Kodak BioMax MR Film. In vitro translation was done by Dr. Agata Starosta of the group of Dr. Daniel Wilson (Gene Center, LMU).

2.6 Bioinformatical methods

Primers for the polymerase chain reaction (PCR) were constructed by using the programs Artemis (Sanger Institute) (Rutherford *et al.*, 2000) and OligoCalc (Oligonucleotide Properties Calculator, http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe, 2007). Predictions of secondary structures were done computer based using the tools PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) (Jones, 1999) and MINNOU (http://minnou.cchmc.org) (Cao *et al.*, 2006). Prediction of signal recognition sites were made computer based using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP) (Petersen *et al.*, 2011). Molecular graphics were constructed with the UCSF Chimera package (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco) (Pettersen *et al.*, 2004). Amino acid alignments were performed using Artemis and the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein&itool=toolbar) (Sayers *et al.*, 2009) for sequences, and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) (Sievers *et al.*, 2011) for the alignment. All Western Blot quantifications were performed by using the software ImageJ (http://rsbweb.nih.gov/ij/) (Schneider *et al.*, 2012).

3. Results

3.1. Importance of YjeK, YjeA and EF-P for *cadBA* expression

The regulation of the acid stress resistance system Cad has been investigated for more than twenty years. Many new discoveries have been made recently (Eichinger *et al.*, 2011; Haneburger *et al.*, 2011; Haneburger *et al.*, 2012; Ruiz *et al.*, 2011), but the system is still not fully understood. Neeley *et al*. reported that chloramphenicol, an inhibitor of translation, abolishes *cadBA* expression, thereby suggesting that de novo synthesis of CadC or another yet unkown protein is required for induction of the Cad system (Neeley & Olson, 1996). To find out, if additional proteins are important for *cadBA* expression, a random mutagenesis approach was employed by Tobias Kraxenberger in his diploma thesis (Kraxenberger, 2006). This experiment revealed YjeK as a new protein involved in the Cad system.

3.1.1. CadA activity in *yjeK*, *yjeA* and *efp* mutants in *E. coli*

cadBA expression shows maximal induction under conditions of low external pH, low oxygen concentration and in the presence of exogenous lysine (Gale, 1946; Sabo *et al.*, 1974). The enzyme of the Cad system, CadA, decarboxylases lysine to CO_2 and cadaverine. The increase in the external pH as a result of CadA activity can be monitored by adding bromocresol purple to the growth medium, which turns to blue at pH values higher than 6.8. At low pH, bromocresol purple gives a yellow colour. This test was previously used by Tobias Kraxenberger to screen a mini-Tn*10* Kan^R library for new players of the Cad system in *E. coli* (Kraxenberger, 2006). Eight of the tested mutants revealed a lysine decarboxylase (CadA) negative phenotype, but only one mutant (P1#36) had intact *cad* genes. The insertion of the mini-Tn*10* Kan^R cassette was mapped downstream of nucleotide 642 in *yjeK* (Kraxenberger, 2006).

To confirm that the negative CadA phenotype is due to disruption of *yjeK* and not to any other effect caused by multiple transposon insertions or secondary effects, a *yjeK* in frame deletion mutant was constructed (Fig. 5A). To avoid polar effects on the neighbouring gene *efp*, only

nucleotides 643 to 1029 of *yjeK* were replaced in MG1655 by a neomycin resistance cassette resulting in strain TK1 (MG1655 *yjeK*$_{643\text{-}1029}$::*npt*) (Kraxenberger, 2006).

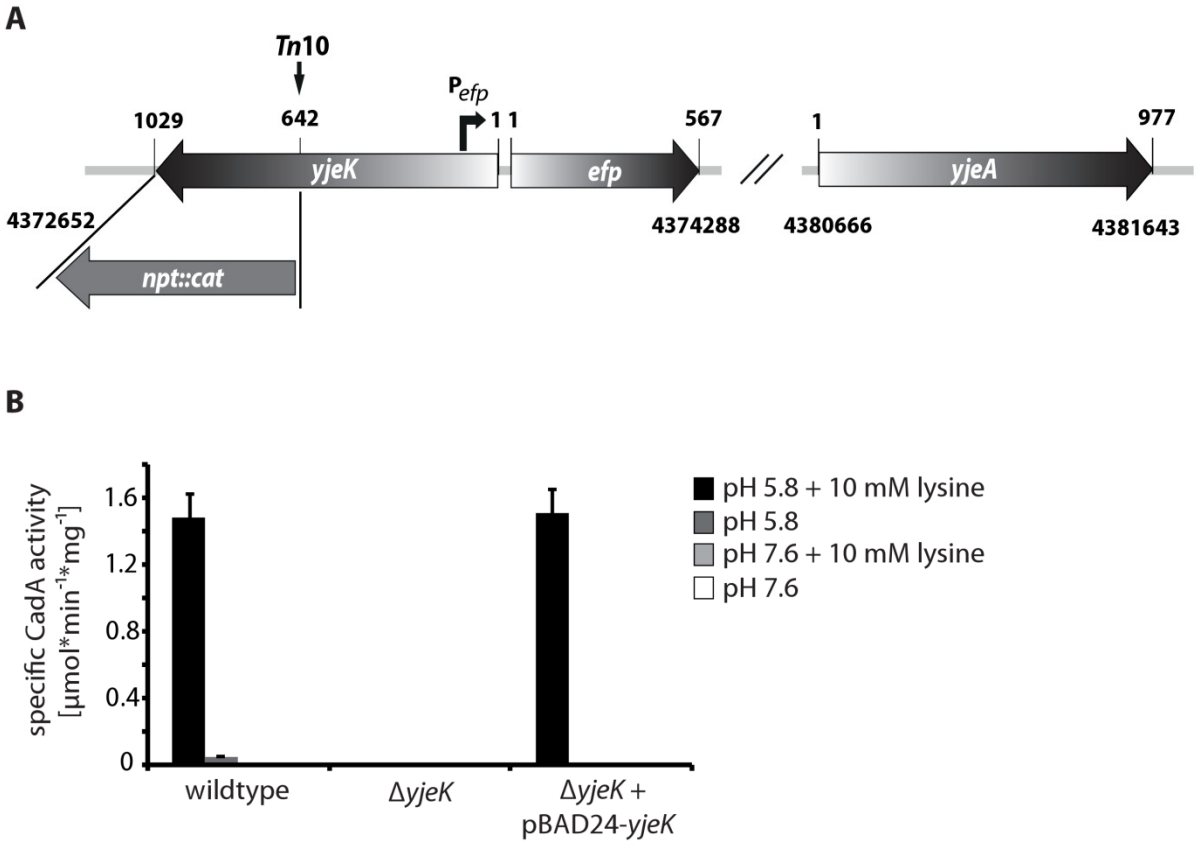


Figure 5: **Genomic characterization and CadA activity in the *yjeK* mutant TK1**.

A) Depiction of the *yjeK* mutation in strain TK1 and genomic localizations of *yjeK*, *efp* and *yjeA*. Corresponding to the transposon mutant P1#36, nucleotides 643 to 1029 of *yjeK* were replaced by a neomycin resistance cassette. The genomic positions of *yjeK*, *efp* and *yjeA* are depicted below the genes. Gene lengths are marked above the genes (in nucleotides).

B) CadA activity in the wildtype (*E. coli* MG1655), TK1 (Δ*yjeK*) and TK1 complemented with plasmid pBAD24-*yjeK*. Cells were cultivated microaerobically in KE minimal medium under inducing (pH 5.8 + 10 mM lysine) and non-inducing (pH 5.8, pH 7.6 + 10 mM lysine, pH 7.6) conditions.

The lysine decarboxylase phenotypes of TK1 and its parental strain MG1655 were determined via measurements of the specific CadA activity. Cells were grown microaerobically in KE minimal medium under inducing (pH 5.8 with 10 mM lysine) and non-inducing (pH 5.8, pH 7.6 with or without 10 mM lysine) conditions. In concert with the transposon mutant, TK1

showed a CadA negative phenotype (Fig. 5B). Complementation of TK1 with plasmid pBAD24-*yjeK* could restore the wildtype phenotype, which revealed CadA activity only under inducing conditions.

It has been recently shown that the elongation factor EF-P (encoded by the *efp* gene) is post-translationally modified by the lysine 2,3-aminomutase YjeK and the truncated lysyl-tRNA synthetase YjeA (Bailly & de Crecy-Lagard, 2010). To test if *yjeA* also affects expression of the Cad system, a *yjeA* deletion mutant was constructed. Similar to TK1, no CadA activity could be detected in strain SU2 (MG1655 *yjeA*::*npt*), when cells were grown in KE medium under inducing conditions. Providing *yjeA* in *trans* in strain SU2 by introducing plasmid pBAD24-*yjeA* led to complementation of the wildtype CadA activity (Fig. 6A).

As EF-P is important for cell viability in *E. coli* (Aoki *et al.*, 1997b), the construction of strain MG1655 Δ*efp* using the pRED/ET method failed. By contrast, *efp* could be deleted in the alternative *E. coli* K-12 strain BW25113 (Baba *et al.*, 2006). To investigate, if a disruption of the *efp* gene leads to greatly reduced CadA activity comparable to that in the *yjeA* and *yjeK* mutants, strains JW4116 and JW4107 harboring a start to stop deletion in *yjeA* and *efp*, respectively, and BW25113 (wildtype) were purchased from the Keio collection (Baba *et al.*, 2006). Additionally, strain SU1 (BW25113 $yjeK_{643\text{-}1029}$::*cat*) was constructed by replacing the 3´ region of *yjeK* by a chloramphenicol resistance cassette in a similar way as for TK1. Strains were grown microaerobically in KE minimal medium under inducing conditions and were further tested on CadA activity. In line with the results obtained for the MG1655 strains, wildtype BW25113 was able to decarboxylate lysine under inducing conditions, whereas the mutant derivatives lacking either *yjeA* or *yjeK* exhibited no significant CadA activity (Fig. 6B). Moreover, a deletion of *efp* led to abolished CadA activity as well. All deletions were complemented by providing the corresponding gene copy in *trans*. As previous results have shown that modification of lysine 34 is crucial for EF-P activity (Navarre *et al.*, 2010; Zou *et al.*, 2011), plasmids pQE70-Eco_*efp* K34A and pQE70-Eco_*efp* K34R encoding inactive EF-P variants with a substitution of lysine 34 either by alanine or arginine were introduced into the *efp* mutant. None of these plasmids could rescue the CadA-negative phenotype of the *efp* mutant (Fig. 6B), thus confirming the results obtained by Navarre *et al.* and Zou *et al.* (Navarre *et al.*, 2010; Zou *et al.*, 2011).

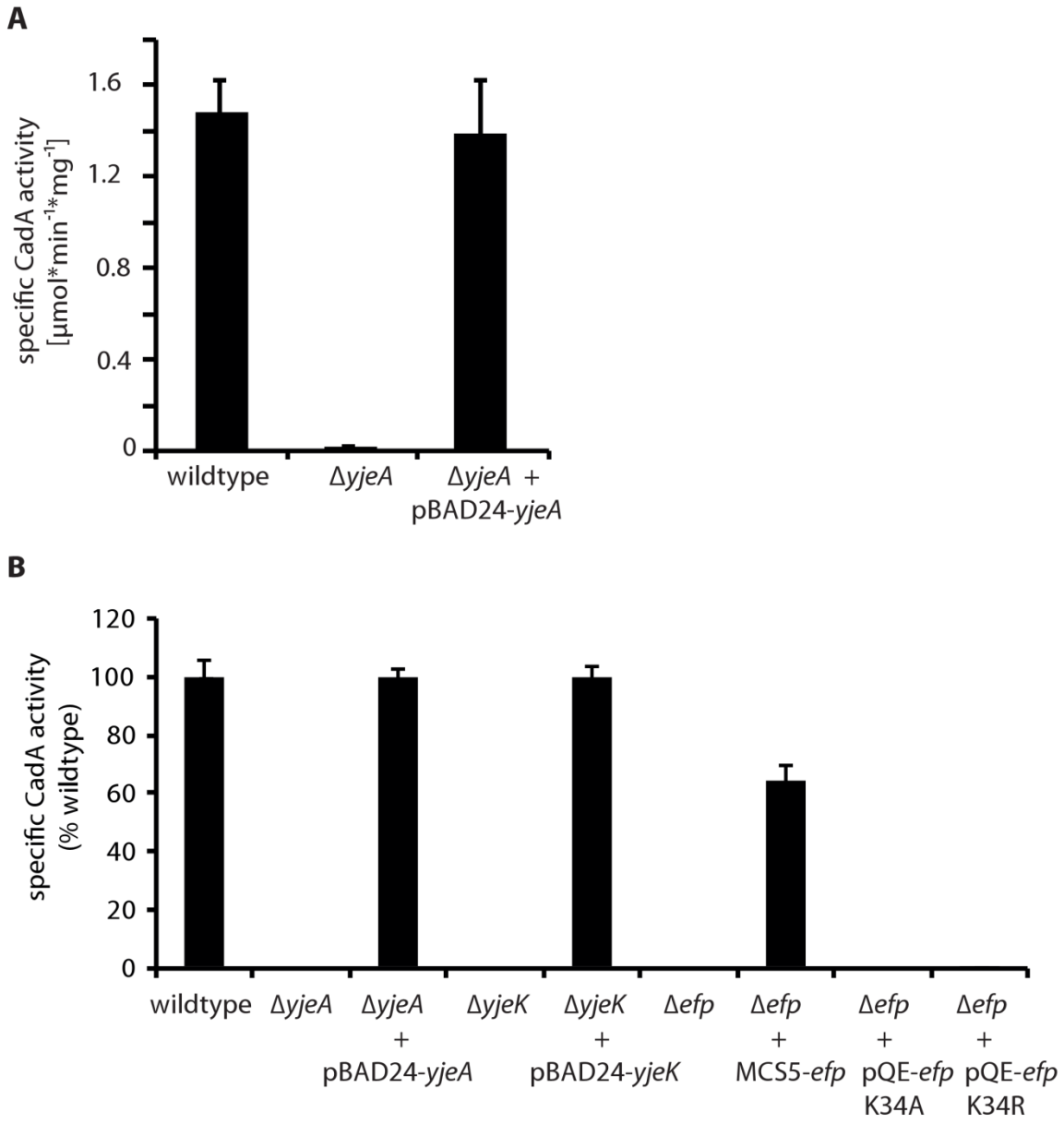


Figure 6: CadA activity in *yjeA*, *yjeK* and *efp* mutants.

A) CadA activity in the *yjeA* mutant. Cells of MG1655 (wildtype), SU2 (MG1655 Δ*yjeA*) and the mutant complemented with pBAD24-*yjeA* were grown microaerobically in KE minimal medium under inducing conditions (pH 5.8 with 10 mM lysine).

B) CadA activity in strains with BW25113 (Keio collection) background. Cells of BW25113 (wildtype), JW4116 (Δ*yjeA*), SU1 (*yjeK*$_{643\text{-}1029}$::*cat*), JW4107 (Δ*efp*) and the mutants provided with the corresponding gene in *trans* were grown microaerobically in KE minimal medium under inducing conditions (pH 5.8 with 10 mM lysine). Plasmids pQE-*efp* K34A and K34R possess a point mutation at the modification site of EF-P.

3.1.2. Complementation of a *yjeK* mutant with KamA of *Clostridium subterminale*

Two different mechanisms have been previously proposed how EF-P is modified (Bailly & de Crecy-Lagard, 2010): Firstly, YjeA activates and transports α-lysine to K34 of EF-P which - after binding to EF-P - is then converted to β-lysine by YjeK. Secondly, YjeK transforms α-lysine to β-lysine, and subsequently YjeA transports the newly built β-lysine to EF-P. Yanagisawa *et al.* supported the first possibility due to the crystal structure of YjeA (Yanagisawa *et al.*, 2010), whereas Gilreath *et al.* and Roy *et al.* demonstrated that β-lysine and not α-lysine is the substrate for YjeA in vitro (Gilreath *et al.*, 2011; Roy *et al.*, 2011). Assuming that the second mechanism is true, YjeK would act as independent supplier of β-lysine. Therefore, a non-related 2,3-aminomutase like KamA of *Clostridium subterminale* should rescue the CadA-negative phenotype of the *yjeK* mutant. KamA shows 33% identity to YjeK and converts (S)-α-lysine to (S)-β-lysine, whereas YjeK catalyzes the isomerization of (S)-α-lysine to (R)-β-lysine (Behshad *et al.*, 2006). To test if KamA can replace YjeK, the *yjeK* gene in strain BL21(DE3) was deleted resulting in strain SU3 (BL21(DE3) $yjeK_{643\text{-}1029}$::*cat*). The mutant was co-transformed with plasmids pAF/*kamA* and pAlter-Ex2/*argU*. As the clostridial codon AGA for arginine is only rarely used in *E. coli*, the second plasmid was needed for expression of the *E. coli argU* gene encoding for the required arginine t-RNA (Ruzicka *et al.*, 2000).

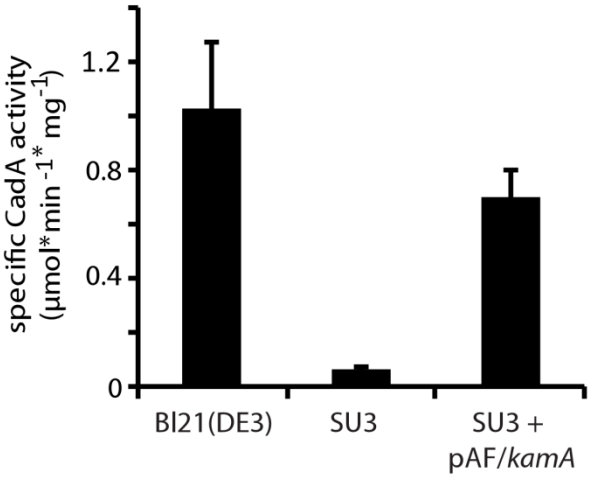


Figure 7: Complementation of the *yjeK* mutant (SU3) with KamA of *C. subterminale*. Cells were grown microaerobically in KE minimal medium under inducing conditions (pH 5.8 with 10 mM lysine).

Interestingly, KamA provided in *trans* to SU3 could restore CadA production, which was abolished in the mutant (Fig. 7). When *yjeK* mutant cells harboring pAF/*kamA* were induced by IPTG, CadA activity was even higher as in the BL21(DE3) wildtype (data not shown). This finding confirms that YjeA transports β-lysine to EF-P for modification, and that YjeK is an independent β-lysine supplier. Additionally, this result indicates that the substrate for YjeA can be (R)- as well as (S)-β-lysine.

3.2. Interaction studies

E. coli YjeK has a size of 38.75 kDa and shows only 0.1% enzymatic activity in comparison to KamA of *Clostridium subterminale* (Behshad *et al.*, 2006). The primary structural difference between these two enzymes is the absence of the C-terminal domain responsible for multimerization in YjeK (Bailly & de Crecy-Lagard, 2010). To find out if YjeK is still able to form dimers, in vivo and in vitro tests were performed. Moreover, the bacterial two hybrid system (BACTH) was employed to investigate the ability of YjeK, YjeA and EF-P to specifically interact with each other or with components of the Cad system.

3.2.1. Dimerization of YjeK in vitro

To characterize YjeK in more detail, the His-tagged protein was purified using Ni^{2+}-NTA affinity chromatography. Purification and the preceding cultivation are described in 2.3.1 and 2.5.1. Immediately after purification, the protein was loaded onto a SDS gel and simultaneously onto a native gel. As YjeK contains a [4Fe-4S] cluster (Behshad *et al.*, 2006), protein eluates showed a yellow coloring after the purification, but the color vanished when the protein was frozen or left on air for several hours (data not shown).
Western Blot analysis was performed using antibodies against His_6-YjeK. Figure 8A depicts the blot with the denaturated gel, where YjeK could be detected at about 40 kDa. By contrast, the native gel clearly showed a band at approximately 80 kDa indicating that YjeK lacking the COOH-terminus of KamA still forms dimers (Fig. 8B).

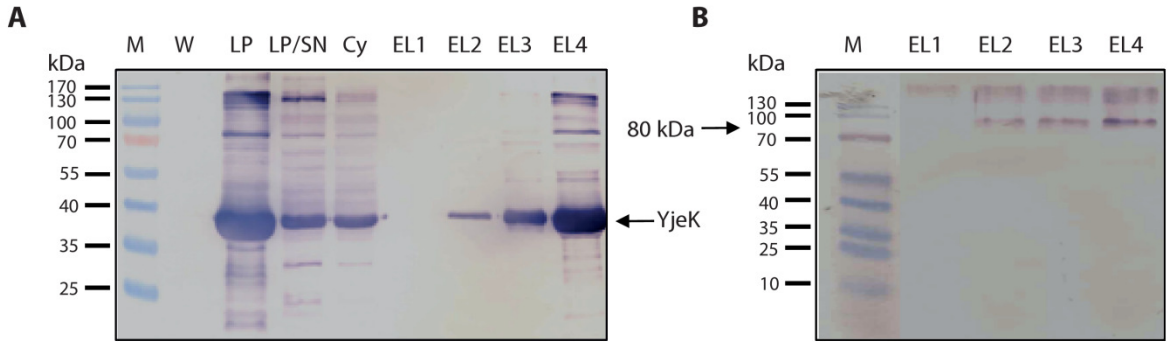


Figure 8: Western Blots using antibodies against purified His_6-YjeK.

A) SDS-PAGE. M: marker (PageRuler Prestained Protein Marker, Fermentas). W: wash fraction. LP: low speed pellet. LP/SN: supernatant of the low speed pellet. Cy: cytosol. EL2-EL4: eluates 2 (5 µg), eluate 3 (25 µg), eluate 4 (46 µg). The protein amount of eluate 1 (EL1) has not been determined.

B) Native gel. M: marker (PageRuler Prestained Protein Marker, Fermentas). Amounts of eluates 2-4 (EL2-4) are equal to the amounts in Fig. 8A. The protein amount of eluate 1 (EL1) has not been determined.

3.2.2. Dimerization of YjeK, YjeA and EF-P with Cad components

Further in vivo interaction studies were performed using the bacterial two hybrid system. To this end, *yjeA*, *yjeK* and *efp* or genes belonging to the Cad system were N- or C-terminally fused to the T25 fragment in vector pKNT-25 or pKT-25, respectively, and additionally C-terminally to the T18 fragment in vector pUT-18C. Only when T18 and T25 fragments come in close proximity due to interaction of the proteins of interest, β-galactosidase activity can be measured. The practical work was done by Christina Krönauer during her bachelor thesis (Krönauer, 2011), the experimental design was done in cooperation with Dr. Jürgen Lassak.

As shown before in vitro, YjeK interacted itself in vivo, too [2,500 Miller Units (MU) and 3,400 MU] (Table 5). Additionally, strong self-interaction was also observed for CadC (3,869 MU), H-NS (3,700 MU and 4,600 MU) and YjeA (1,100 MU and 2,600 MU). This was expected as previous studies reported dimerization/multimerization of these proteins (Eichinger *et al.*, 2011; Smyth *et al.*, 2000; Ueguchi *et al.*, 1996; Yanagisawa *et al.*, 2010).

Moreover, the lysine permease LysP also showed dimerization. This ability for self-interaction (900 MU) is in good agreement with earlier findings for the Na^+/H^+ antiporter

NhaA (Hilger *et al.*, 2007; Williams, 2000; Williams *et al.*, 1999). Furthermore, it was previously suggested that CadC interacts with LysP (Tetsch *et al.*, 2008). Indeed, high β-galactosidase activities were observed using CadC and LysP containing plasmids (3,000 MU) indicating stable hetero-dimerization of both proteins in vivo.

Table 5: Bacterial two hybrid studies. *cadC*, *lysP*, *hns*, *yjeA*, *yjeK* and *efp* were cloned into the vectors of the bacterial two hybrid system by N-terminal (pKNT-25) or C-terminal (pKT-25, pUT-18C) fusion to the T25 or T18 fragment of the adenylate cyclase of *Bordetella pertussis*. β-Galactosidase activity resembles the strength of interaction: high (++, over 800 MU), low (+, over 200 MU), no (-, under 200 MU) interaction.

		pUT-18C					
		CadC	**LysP**	**H-NS**	**YjeA**	**YjeK**	**EF-P**
PK(N)T-25	**CadC-N**	++	++	-	-	-	-
	LysP-N	++	++	-	-	-	-
	LysP-C	++	++	-	-	-	-
	H-NS-N	-	-	++	-	-	-
	H-NS-C	-	-	++	-	-	-
	YjeA-N	-	-	-	++	-	+
	YjeA-C	-	-	-	++	-	-
	YjeK-N	-	-	-	-	++	-
	YjeK-C	-	-	-	-	++	-
	EF-P-N	-	-	-	-	-	-
	EF-P-C	-	-	-	-	-	+

Additionally, YjeK and EF-P or YjeK and YjeA, respectively, showed no interactions at all. By contrast, YjeA interacted with EF-P, when strains harbored plasmids pK(N)T25-*yjeA* and pUT18C-*efp* (300 MU). In line with this, co-crystals of YjeA in complex with EF-P suggested stereospecific interaction of these proteins in vivo (Yanagisawa *et al.*, 2010). These results support once again that YjeK first converts α-lysine to β-lysine, which is then activated and transported to EF-P by YjeA (Bailly & de Crecy-Lagard, 2010).

It would be conceivable that also CadC is post-translationally modified, as exchanges of lysines against alanines or arginines revealed lysine residue 95 as possible candidate for modification in CadC (Fig. A1). However, neither CadC and H-NS nor LysP showed any specific interaction with YjeA or YjeK (Table 5) making a post-translational modification of other proteins by YjeA and YjeK rather unlikely.

3.3. Determining the target of EF-P in the Cad system

3.3.1. *cadBA* expression in *yjeA*, *yjeK* and *efp* mutants

According to the results described above, a deletion of *efp* or any of its modifiers leads to a negative CadA activity. No direct target for EF-P has ever been identified before, therefore it was suggested that EF-P as well as eIF5A are not essential for global protein synthesis, but are required for only a limited number of mRNAs (Kang & Hershey, 1994; Yanagisawa *et al.*, 2010; Zou *et al.*, 2011). To this end, it was further investigated, if EF-P influences the translation of one or more Cad proteins or if another yet unknown protein is involved.

3.3.1.1. Expression of *lacZ* under control of the *cadBA* promoter

The phenotypical analysis of the *yjeK*, *yjeA* and *efp* mutants described above focus on CadA activity, but does not answer the question which protein is directly influenced by active EF-P. Hence, the observed effect could result from reduced CadA translation, activity or from reduced *cadA* transcription. To address this question, the *cadBA* operon was replaced by a promoterless *lacZ* in strain MG1655 Δ*lacZ*. The mutation resulted in strain MG-CR (MG1655 Δ*lacZ* Δ*cadBA cadBA*::*lacZ*) containing a P_{cadBA}-*lacZ* fusion. Additionally, gene *yjeK* or *yjeA* was deleted by an exchange with a kanamycin cassette resulting in strain MG-CR-*yjeK* or MG-CR-*yjeA*. In the case of reduced CadA translation or activity in the mutants, one would expect to observe *lacZ* expression, but in the case of reduced *cadBA* transcription, the expression of *lacZ* should also be lowered.

All strains were incubated microaerobically in KE medium under inducing (pH 5.8 with 10 mM lysine) or non-inducing (pH 5.8) conditions and cells were harvested at their exponential growth phase (OD_{600} = 0.3 - 0.8). Under inducing conditions, *lacZ* was only expressed in the parental strain MG-CR, but not in the *yjeK* and *yjeA* mutants (Fig. 9). The same result was achieved with strains MG-CL-1 (*cadA*::*lacZ*), MG-CL-1-*yjeA* (*cadA*::*lacZ* Δ*yjeA*) and MG-CL-1-*yjeK* (*cadA*::*lacZ* Δ*yjeK*) (Fig. A2-A). By contrast, the two mutants harboring plasmids pBAD24-*yjeA* or pBAD24-*yjeK* encoding the missing genes in *trans* showed β-galactosidase activity values similar to their parental strains (Fig. 9). This result clearly shows that modified EF-P is crucial for *cadBA* expression.

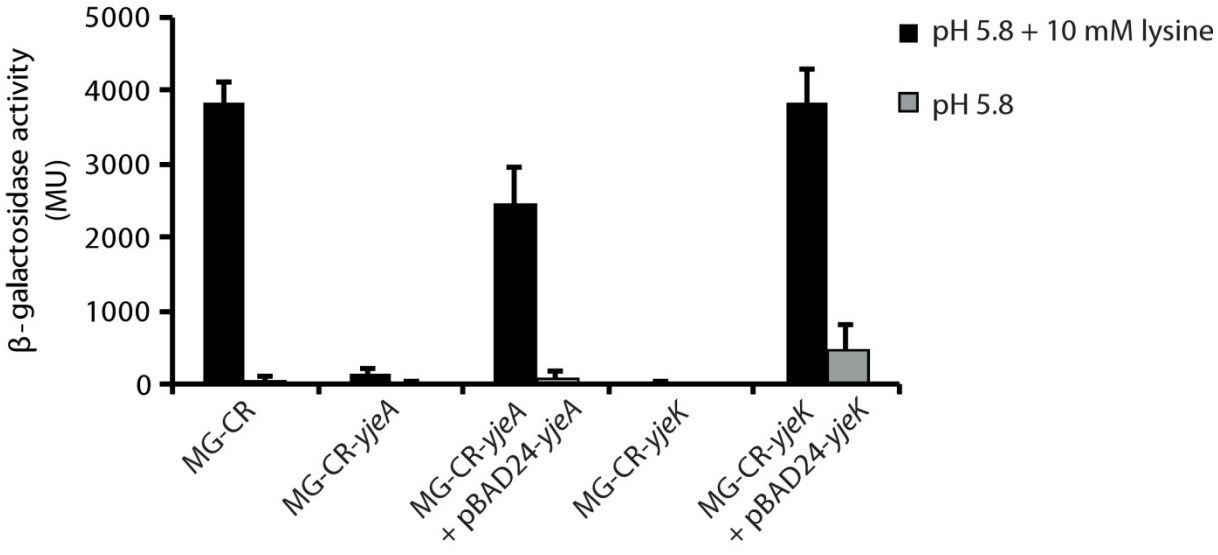


Figure 9: **Expression of P_{cadBA}-*lacZ* in *yjeA* and *yjeK* mutants.** Strains MG-CR (MG1655 Δ*lacZ* Δ*cadBA cadBA*::*lacZ*), MG-CR-*yjeA* (Δ*yjeA*), MG-CR-*yjeK* (Δ*yjeK*) and the complemented mutants were grown microaerobically in KE medium under inducing (pH 5.8 with 10 mM lysine) and non-inducing (pH 5.8) conditions.

3.3.1.2. *cadB* and *cadA* transcription in *yjeK*, *yjeA* and *efp* mutants

To investigate, if the reduced β-galactosidase activity in the *yjeK* and *yjeA* mutants attributes to an impaired LacZ translation or to reduced *cadBA* expression, transcript levels of the *cadBA* operon were examined in wildtype MG1655 and in TK1 ($\Delta yjeK_{643\text{-}1029}$). For this experiment, cells were grown in KE medium aerobically under non-inducing conditions (pH 7.6) at 37 °C to an OD_{600} of 1. Subsequently, cells were shifted to inducing conditions (KE minimal medium, pH 5.8 with 10 mM lysine) in a 5 liter fermenter. Samples for RNA isolation were taken 0 / 15 / 20 / 25 min after the shift. The isolated RNA was used to perform Northerns Blot with probes against *cadA* and *cadB*. For control, also a probe against *cadC* was employed.

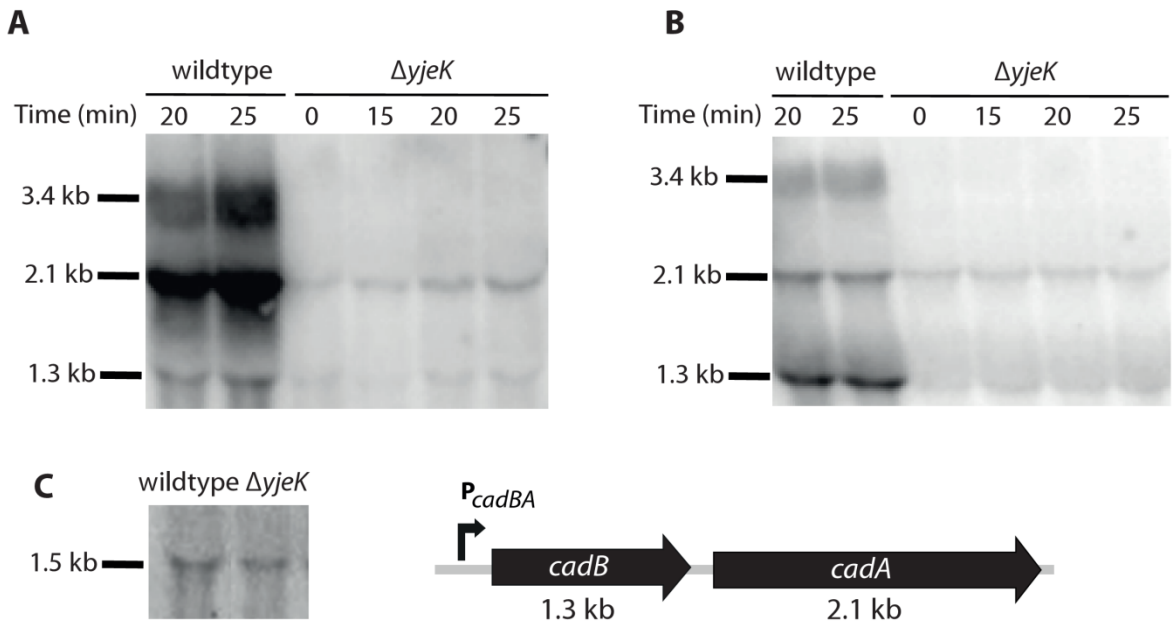


Figure 10: Northern Blot analysis with probes against *cadA, cadB* and *cadC*. Cells of MG1655 (wildtype) and TK1 (Δ*yjeK*$_{643\text{-}1029}$) were first grown under non-inducing conditions (pH 7.6) and then shifted to inducing conditions (pH 5.8 with 10 mM lysine) in KE minimal medium. Time is given in minutes after the shift. Radioactive probes were directed against *cadA* (**A**), *cadB* (**B**) and *cadC* (**C**). For *cadC* as control, only time point 20 (min) is shown. The figure depicts the *cadBA* operon.

As expected, the two *cad* genes were transcribed in the wildtype strain and revealed an expression peak at 25 min (Fritz *et al.*, 2009). It has been previously published that mRNAs of operons are preferably degraded in 5´ to 3´ direction, and that the half-life of *cadB* is very short in comparison to other mRNAs (Selinger *et al.*, 2003). This finding is well reflected by the higher amount of *cadA* than *cadB* in the Northern Blot (Fig. 10A, B). Accordingly, β-galactosidase activities in cells of strain MG-CL-1 (*cadA*::*lacZ*) were approximately two times higher than in cells of strain MG-CR (*cadBA*::*lacZ*) cells, when they were cultivated under inducing conditions (Fig. 9 and Fig. A2-A). An additional promoter upstream of *cadA* was excluded (Fig. A2-B).

Interestingly, the amount of both *cadA* and *cadB* mRNA was highly reduced in TK1 (Fig. 10A, B). Transcription of *cadC* was low, but no difference could be detected between the wildtype and the mutant (Fig. 10C).

Additionally, *cadBA* expression of strains BW25113, JW4094 (Δ*cadC*), JW4116 (Δ*yjeA*), SU1 (Δ*yjeK*$_{643\text{-}1029}$) and JW4107 (Δ*efp*) were analyzed from induced cells by quantitative real time PCR (qRT-PCR). As in the Northern Blot, a strong induction could be measured for

cadA mRNA (10,000 fold) as well as *cadB* mRNA (5,000 fold) in the wildtype (Fig. 11). By contrast, expression of the *cad* genes was highly reduced in the Δ*yjeA*/*yjeK*/*efp* mutants indicating that active EF-P is crucial for *cadBA* expression.

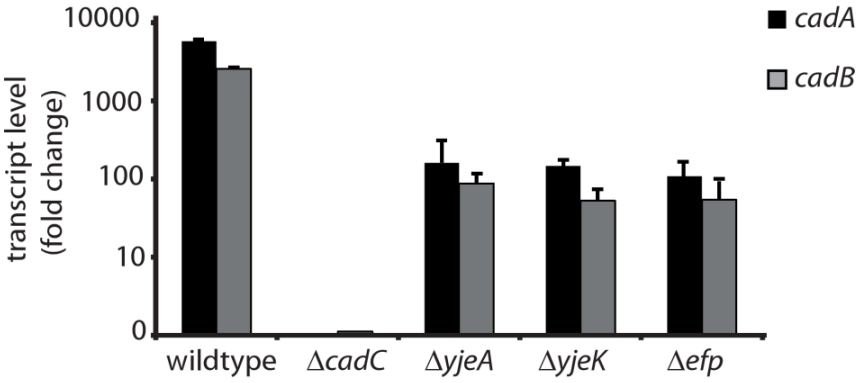


Figure 11: *cadA* and *cadB* transcript levels in the wildtype and *yjeA*, *yjeK*, *efp* mutants. Transcript levels were determined by qRT-PCR in BW25113 (wildtype), JW4094 (Δ*cadC*), JW4116 (Δ*yjeA*), SU1 (Δ$yjeK_{643\text{-}1029}$) and JW4107 (Δ*efp*). JW4094 served as negative control. Cells were grown and harvested under inducing conditions (KE minimal medium, pH 5.8 + 10 mM lysine).

3.3.2. CadC as possible target of EF-P

The phenotypic analysis of the *yjeA*, *yjeK* and *efp* deletion mutants aimed on *cadBA* expression, but left the question open which protein of the Cad-regulatory system is EF-P dependent. As CadC acts as positive regulator of the *cad* operon, further experiments focused on *cadC* translation.

3.3.2.1. CadC protein levels in the wildtype and *yjeA*, *yjeK*, *efp* mutants

In a next experiment, the amount of CadC in *E. coli* wildtype cells was determined and compared to mutants lacking *yjeA*, partially *yjeK* or *efp*. As CadC is not an abundant protein in the cell, its detection in Western Blot analysis can be difficult (Tetsch *et al.*, 2008). Therefore, the *cadC* encoding plasmid p5C (pBBR1-MCS5-*cadC*) was used to increase the signal intensitiy of CadC. The protein was left under control of its native promoter. Thus, a

potential increase in signal intensity should be caused only by an increase of the plasmid copy number, which was reported to be 30-40 copies per cell (Antoine & Locht, 1992), but not by different transcription levels. To this end, *E. coli* BW25113, JW116 (Δ*yjeA*), SU1 (*yjeK*$_{643\text{-}1029}$) and JW4107 (Δ*efp*) harboring p5C, respectively, and JW4095 (Δ*cadC*) as a negative control were grown aerobically in KE medium to exponential phase (OD_{600} = 0.4-0.6) under non-inducing conditions (pH 7.6). Subsequently, cells were shifted to inducing conditions (pH 5.8, 10 mM lysine, without shaking) for 1 h. After preparation of the membrane vesicles, the total protein amount was determined, and 150 µg per sample were loaded onto a SDS gel. CadC accumulation was measured by Western Blot analysis using CadC specific antibodies, and the CadC signal intensity was analyzed using the program ImageJ. Strikingly, the Western Blot data clearly showed that the CadC amount is significantly reduced to 26%, 25% and 17% of wildtype CadC in cells harboring a deletion of *yjeA, yjeK* and *efp*, respectively (Fig. 12).

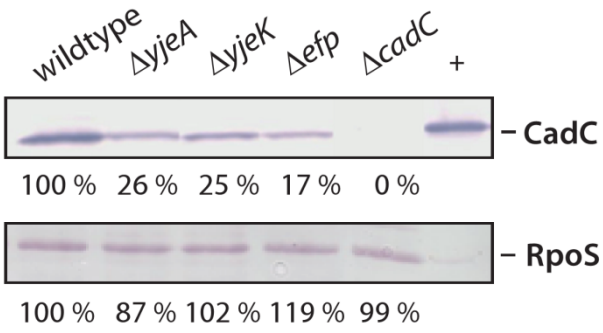


Figure 12: CadC levels in the wildtype and *yjeA*, *yjeK*, *efp* mutants. Cells were grown and harvested under inducing conditions (KE minimal medium, pH 5.8 + 10 mM lysine). The Western Blot was performed using antibodies against CadC and RpoS, respectively. The RpoS levels served as control.

As a control, the protein levels of the alternative sigma factor RpoS were determined. If the *cadC* mRNA is a specific target of EF-P, RpoS protein levels should not differ in the wildtype and the mutants. Indeed, the amount of RpoS was very similar in logarithmically grown cells of the wildtype and the mutants (Fig. 12).

3.3.2.2. *cadC* transcription in the wildtype and *yjeA*, *yjeK*, *efp* mutants

To exclude an indirect effect on *cadC* transcript levels in Δ*yjeK*$_{643\text{-}1029}$, Δ*yjeA* and Δ*efp* mutants caused by transcriptional regulation, the *cadC* mRNA levels were compared by qRT-PCR. Cells of wildtype BW25113, JW116 (Δ*yjeA*), SU1 (Δ*yjeK*$_{643\text{-}1029}$), JW4107 (Δ*efp*) and JW4095 (Δ*cadC*) were grown aerobically in KE medium, pH 7.6, to an OD_{600} of 0.4 – 0.6. Subsequently, cells were shifted to inducing conditions (KE medium, pH 5.8 with 10 mM lysine) and were incubated microaerobically for 25 min. Samples were then taken to isolate RNA for the qRT-PCR analysis.

No difference in the *cadC* transcription levels could be observed between the tested strains indicating that the transcription of *cadC* is unaffected by EF-P (Fig. 13A).

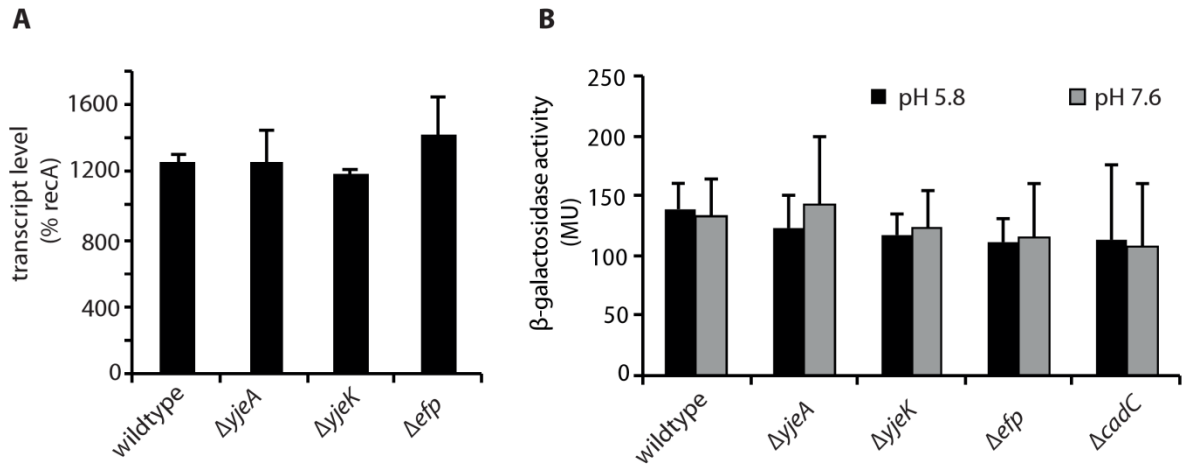


Figure 13: *cadC* transcription in the wildtype and *yjeA*/*yjeK*/*efp* mutants. Transcript levels were determined either by qRT-PCR (**A**) or by β-galactosidase activity using a transcriptional P_{cadC}::*lacZ* fusion in plasmid p3LC-TF (**B**).

According to these data, a transcriptional fusion of the *cadC* promoter to *lacZ* in plasmid pBBR-MCS3 was constructed. Strains BW113, JW4115, SU1 and JW4107 harboring the resulting plasmid p3LC-TF revealed no remarkable variations in β-galactosidase activity in the wildtype compared to the mutants, either under non-inducing conditions (LB medium, pH 7.6) nor under inducing conditions (LB medium, pH 5.8) (Fig. 13B).

3.4. The role of EF-P in translation

It was previously shown that EF-P stimulates peptide bond formation between small amino acids like glycine, but not between bulky or charged amino acids like phenylalanine and the ribosome bound fMet-tRNA in vitro (Aoki *et al.*, 1997a; Ganoza & Aoki, 2000; Glick *et al.*, 1979). Thus, it was suggested that EF-P could be a helper protein of the ribosome driven first peptide bond synthesis (Glick *et al.*, 1979). By contrast, other investigations to eIF5A pointed more into the direction that EF-P and its eukaryotic orthologue act as translation elongators (Gregio *et al.*, 2009; Saini *et al.*, 2009). CadC as first direct target of EF-P opened now the possibility to shed light on this controverse discussion.

3.4.1. EF-P as possible helper protein for first peptide bond formation

To focus on the question, if EF-P helps the ribosome to form the first peptide bond, wildtype *cadC* and its native promoter were cloned into plasmid pBBR-MCS5, thus giving plasmid p5C. Additionally, the second amino acid of CadC (glutamine) was exchanged by a glycine or phenylalanine in plasmids p5C-Q02G and p5C-Q02F. Strains MG-CL-123 (*cadC*::*npt* P_{cadBA}-*lacZ*), MG-CL-12-*yjeA* (*yjeA*::*npt* P_{cadBA}-*lacZ*) and MG-CL-12-*yjeK* (*yjeK*$_{643\text{-}1029}$::*npt* P_{cadBA}-*lacZ*) harboring one of the plasmids, respectively, were grown microaerobically at 37 °C in KE medium under inducing conditions (pH 5.8 with 10 mM lysine). Cells were harvested at their exponential growth phase (OD_{600} of 0.4 – 0.6), and subsequently β-galactosidase activity was measured. In the case of EF-P being important for first peptide bond synthesis, EF-P would only be needed when the second amino acid is small like glycine, but not when it is bulky like phenylalanine. Thus, one would expect wildtype like *cadBA* expression in the *yjeA* and *yjeK* mutants, when they harbor plasmid p5C-Q02F, but no expression with plasmid p5C-Q02G. Indeed, *yjeA* and *yjeK* mutants showed an approximately three times higher β-galactosidase activity with plasmid p5C-Q02F than with plasmid p5C encoding wildtype *cadC* (Fig. 14A). However, β-galactosidase activities obtained with plasmid p5C-Q02F only reached 14% (Δ*yjeA*) and 11% (Δ*yjeK*$_{643\text{-}1029}$) of the MG-CL-123 (Δ*cadC*) values. Furthermore, strains harboring plasmid p5C-Q02G revealed not only very low β-galactosidase activities in the *yjeA* and *yjeK* mutants, but also in the MG-CL-123 strain.

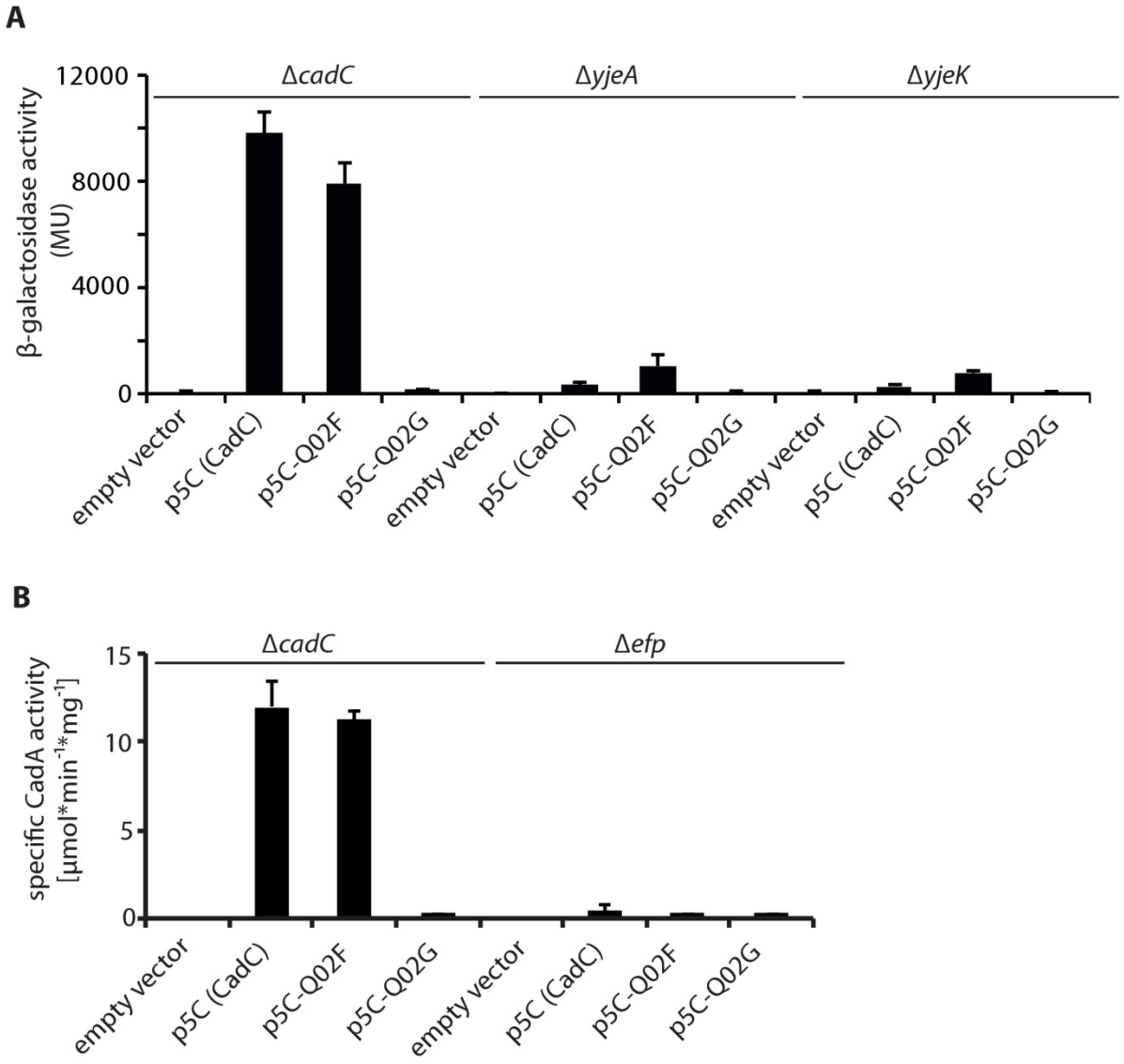


Figure 14: Influence of the second amino acid of CadC on *cadBA* expression.

A) Determining P_{cadBA}-*lacZ* expression by β-galactosidase activity in Δ*cadC,* Δ*yjeA* and Δ*yjeK*$_{643\text{-}1029}$ mutants triggered by different CadC variants with glutamate, phenylalanine or glycine at amino acid position two. Mutants derived from strain MG-CR were cultivated in KE minimal medium under inducing conditions (pH 5.8, 10 mM lysine).

B) CadA activity in JW4095 (Δ*cadC*) and JW4107 (Δ*efp*) harboring the same CadC variants as described in A.

Accordingly, plasmids p5C, p5C-Q02F and p5C-Q02G expressed in JW4107 (Δ*efp*) and JW4095 (Δ*cadC*) led to similar results, when the specific CadA activity was measured. The Δ*efp* mutant containing plasmid p5C-Q02F could not achieve wildtype like CadA activity,

whereas plasmid p5C-Q02G led to minimal β-galactosidase activity in both tested strains (Fig. 14B).

In a parallel experiment it was investigated, if EF-P could trigger first peptide bond formation in LacZ synthesis. Therefore, *lacZ* was set under control of the *cadC* promoter in plasmid pBBR-MCS3-NP-CadC (p3LC) to have similar transcriptional regulation as *cadC*. Additionally, the second amino acid of LacZ, which is normally represented by a histidine in plasmid p3LC, was exchanged by a glycine, proline, leucine, alanine, methionine, phenylalanine or glutamine. The resulting plasmids p3LC-TL02-Gly, -Pro, -Leu, -Ala, -Met, -His, -Phe and -Gln were transformed into strains BW25113 and JW4107 (Δ*efp*), respectively. Cells were grown microaerobically in LB medium at 37 °C, until they were harvested at their exponential growth phase. Subsequently, β-galactosidase activity was measured. In contrast to the in vitro experiments of Glick *et al.* (Glick *et al.*, 1979), β-galactosidase activities in wildtype and Δ*efp* cells varied with none of the plasmids (Fig. 15A). Therefore, the results obtained with *cadC* and *lacZ* exclude a role of EF-P in first peptide bond formation in vivo.

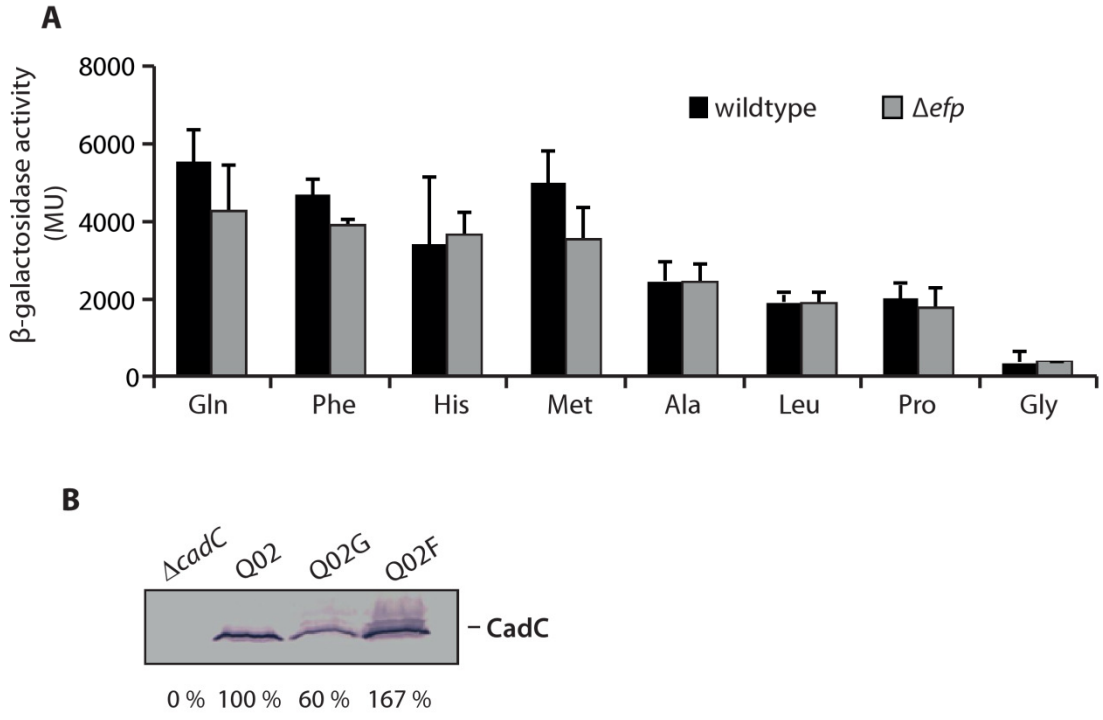


Figure 15: Influence of the second amino acid on the protein amount.

A) Comparison of the expression of various P_{cadC}-*lacZ* variants in the wildtype (BW25113) and JW4107 (Δ*efp*). The second amino acid of LacZ was exchanged by different amino acids. Strains were cultivated in LB medium.

B) Comparison of the CadC amounts of different CadC variants in JW4095 (Δ*cadC*). Cells were cultivated in LB medium. The CadC signal intensity was analyzed by using the program ImageJ.

As demonstrated before, expression levels of proteins highly depend on the nature of the second amino acid. To confirm that a glycine at amino acid position two reduces the translation rate in *E. coli* proteins, whereas phenylalanine enhances it, wildtype BW25113 strain harboring plasmid pBBR-MCS5 (empty plasmid), p5C (wildtype CadC), p5C-Q02F or p5C-Q02G and strain JW4095 (Δ*cadC*) were cultivated aerobically in 500 ml LB medium. When cells have reached an OD_{600} of 1, membrane vesicles were prepared. 150 µg of the total protein amount per lane was loaded onto a SDS gel, and a Western Blot was performed using CadC specific antibodies. Indeed, the CadC amount in the strain harboring p5C-Q02G reached only 60% in comparison to the strain harboring p5C, whereas plasmid p5C-Q02F lead to 1.7 times more CadC as in the wildtype strain (Fig. 15B).

3.4.2. EF-P as specific translation elongation factor of CadC

Earlier reports suggested that eIF5A plays a role in translation elongation rather than translation initiation (Gregio *et al.*, 2009; Saini *et al.*, 2009; Zanelli *et al.*, 2009). To investigate if EF-P acts as translation elongation factor of CadC, the promoter and 2, 30, 66, 100, 131 and 158 codons of *cadC* were fused to *lacZ* in vector pBBR-MCS3 resulting in plasmids p3LC-TL02, -TL30, -TL66, TL100, -TL131 and -TL158, respectively. Thus, various lengths of the DNA-binding domain and the linker region of CadC were comprised in the fusion proteins (Fig. 16A). The plasmids were transformed into wildtype cells and Δ*efp* cells. Cells were grown in LB medium at 37 °C, harvested at exponential growth phase and the β-galactosidase activities of the wildtype (efp^+) and the mutant (efp^-) were compared.

No difference in β-galactosidase activity could be observed between wildtype and mutant cells harboring plasmids p3LC-TL02, -TL30, -TL66 and -TL100 indicated by an efp^-/efp^+ ratio of ~1.0 (Fig. 16B). By contrast, the β-galactosidase activities of TL131 and TL158 were significantly decreased in the *efp* mutant strain, as exemplified by a decreased ratio of 0.3 and 0.15, respectively. This observation provided the first hint that EF-P dependent translation of CadC relies on a motif located between amino acids 108 and 158.

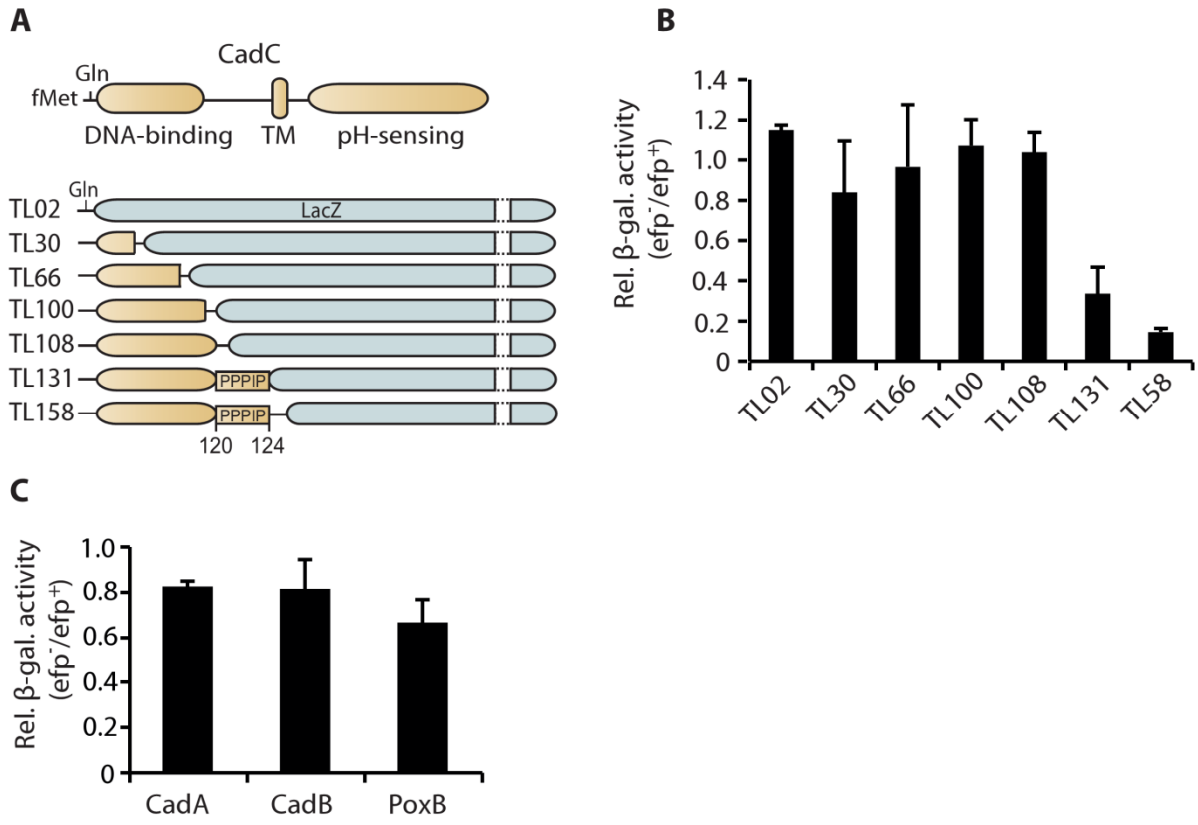


Figure 16: EF-P as specific translation elongation factor of CadC.

A) Schematic depicting the domain structure of full-length CadC in comparison with CadC'-LacZ hybrids.

B) The β-galactosidase activities of CadC'-LacZ (TL02-TL158) hybrids from (A) were determined in JW4107 (*efp*$^-$) and BW25113 (*efp*$^+$) as ratio (*efp*$^-$/*efp*$^+$). Strains were cultivated in LB medium.

C) Effect of EF-P on translation of CadA, CadB and PoxB. The β-galactosidase activities of a translational fusion of full length *cadA*, *cadB* or *poxB*, respectively, and *lacZ* were measured in *efp*$^-$ and *efp*$^+$ strains as ratio (*efp*$^-$/*efp*$^+$). Strains were cultivated in LB medium.

To address the question if not only CadC, but also CadA und CadB are targets of EF-P, translational *lacZ* fusions of full-length *cadA* and *cadB* were designed. Additionally, the pyruvate oxidase PoxB was tested. It has previously been shown that a mutation of *poxA* (synonymous with *yjeA*) reduces the pyruvate oxidase activity (Kaniga *et al.*, 1998), which could be caused by diminished translation of *poxB*. The *cadA-lacZ*, *cadB-lacZ and poxB-lacZ* fusions were set under control of the *cadC* promoter to maintain equal transcriptional regulation. The plasmids were transformed into wildtype and Δ*efp* cells and after growth in LB medium, the β-galactosidase activities of *efp*$^+$ and *efp*$^-$ cells were compared. No

remarkable difference in β-galactosidase activities could be observed in any of the LacZ-hybrid proteins (Fig.16C). This result not only confirms that CadA, CadB and PoxB are no targets for EF-P, but also corroborates strongly that EF-P is not a general translation factor, but is rather needed for the translation elongation of a small subset of proteins as suggested by Navarre *et al*. (Navarre *et al.*, 2010).

3.5. Searching for the EF-P signal sequence

3.5.1. The CadC proline cluster and its role for EF-P dependency

CadC belongs to the family of ToxR-like transcriptional activators (Watson *et al.*, 1992). These proteins are characterized by a conserved modular composition consisting of a N-terminal cytoplasmic winged helix-turn-helix DNA-binding motif followed by a single transmembrane domain and a C-terminal periplasmic sensing domain (Egan *et al.*, 2002, Hase & Mekalanos, 1998; Merrell & Camilli, 2000; Miller *et al.*, 1987) (Fig. 17A). A cytoplasmic putative unstructured region between aa 104 and aa 158 interconnects the input and output domains. As shown before, impaired translation elongation of CadC in *efp* mutant strains is restricted to this part (Fig. 16B). This raises the question for a specific recognition sequence. Interestingly, a cluster of four prolines (Pro120-Pro121-Pro122-Ile123-Pro124) was found in this region (Fig. 17B). Three of the prolines are consecutive, the last one is separated by an isoleucine. When the unstructured region of *E. coli* and four additional species of the *Enterobacteriaceae* were blasted, the proline cluster could be observed in all of these species (Fig. 17B).

Of the 20 canonical amino acids, proline is very special in that it has a secondary α-amino group. Accordingly, proline is a particularly poor acceptor during peptide-bond formation (Muto & Ito, 2008; Pavlov *et al.*, 2009). Since EF-P has been shown to stimulate peptide-bond formation in vitro (Glick *et al.*, 1979; Glick & Ganoza, 1975), it was hypothesized that EF-P might support translation by overcoming the detrimental effect of the consecutive prolines and releasing ribosomal stalling. To test this hypothesis, two in vivo approaches were used:

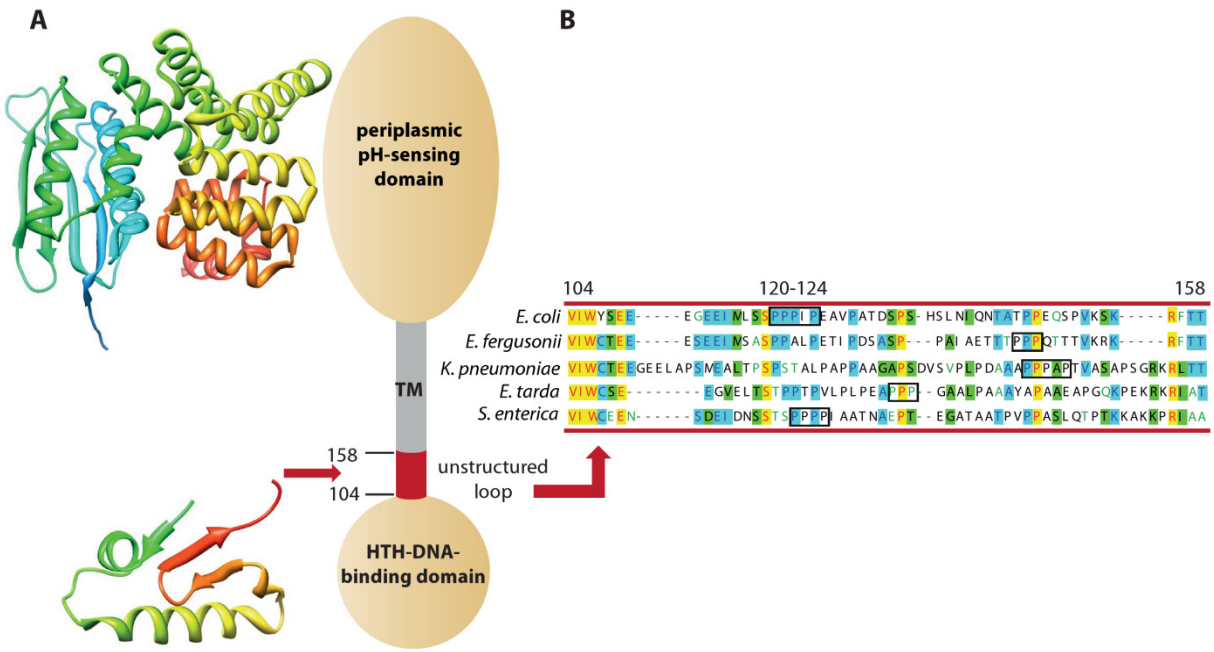


Figure 17: The proline cluster in the unstructured loop region of CadC.

A) CadC domains. CadC consists of a N-terminal helix-turn-helix (HTH) motif for DNA binding and a periplasmic domain for pH sensing. Domain models were based on the crystal structures of the periplasmic domain of *E. coli* CadC (PDB code: 3LY7) (Eichinger *et al.*, 2011) and of the DNA-binding domain of *E. coli* PhoB (PDB code: 1QQI) (Blanco *et al.*, 2002), which shows high similarity to CadC. TM (grey): transmembrane domain. Dark red: unstructured loop region.

B) Amino acid alignment of the unstructured region of CadC in some selected enterobacteria (*E. coli, Escherichia fergusonii, Klebsiella pneumonia, Edwardsiella tarda* and *Salmonella enterica*). Sequences were compared using Clustal Omega (Sievers *et al.*, 2011). Proline clusters are marked by black boxes.

Firstly, each single proline of full length CadC was exchanged in plasmid p5C (pBBR-MCS5 P_{cadC}-*cadC*) by alanine resulting in plasmids p5C-P120A, p5C-P121A, p5C-P122A and p5C-124A. Additionally, the complete proline cluster was substituted in plasmid p5C-P/A. Strain JW4107 (Δ*efp*) harboring plasmids p5C-P120A, p5C-P121A, p5C-P122A and p5C-124A, respectively, and the BW25113 wildtype were cultivated in LB medium and harvested at an OD_{600} of 1. After purification of the membrane vesicles, 150 µg were loaded onto a SDS gel and the CadC amount was compared. Strikingly, CadC with P/A substitutions expressed in the Δ*efp* mutant reached nearly fully (p5C-P/A) or partially (p5C-P120A, p5C-P121A, p5C-P122A) wildtype like protein amounts (Fig. 18A) confirming that the three consecutive prolines are crucial for EF-P dependency. By contrast, mutants harboring plasmid p5C-P124A

remained EF-P dependent. This result indicates that three prolines in a row are sufficient for the EF-P dependent ribosomal stalling.

Secondly, the CadC´-LacZ-translational fusion TL158 in plasmid p3LC-TL158 was employed to generate proline to alanine derivatives as well and to depict the translational rate as function of relative β-galactosidase activity. The β-galactosidase activities of the wildtype (*efp*$^+$) and the mutant (*efp*$^-$) were compared and indicated by an *efp*$^-$/*efp*$^+$ ratio. In accordance to Fig. 16B, the Δ*efp* mutant harboring plasmid p3LC -TL158 showed a highly decreased β-galactosidase activity (0.13) (Fig. 18B). By contrast, constructs p3LC-TL158-P121A and p3LC -TL158-P/A resulted in an EF-P independent phenotype with a ratio of 0.75 and 0.85, respectively. Comparable to the Western Blot results, mutants harboring p3LC-TL158-P120A (0.3) or p3LC-TL158-P122A (0.4) were partially EF-P independent, the mutant harboring p3LC-TL158-P124A remained fully EF-P dependent (0.13). These results confirm again the importance of three consecutive prolines for EF-P dependent translation. Furthermore, this hypothesis is supported by the fact that an interruption of consecution in p3CL-TL158-P121A led to a stronger EF-P independent phenotype compared to p3LC-TL158-P120A and p3LC-TL158-P122A (Fig. 18B).

To examine if the disruption of the proline cluster results not only in higher CadC amounts, but also in increased CadA activities in the Δ*efp* mutant, strain JW4095 (Δ*cadC*) and JW4107 (Δ*efp*) harboring plasmids p5C-P120A, p5C-P121A, p5C-P122A, p5C-124A and p5C-P/A were cultivated microaerobically in pH 5.8 buffered KE medium. Cells were harvested at their exponential growth phase and the β-galactosidase activities between *efp*$^+$ and *efp*$^-$ cells were compared. Remarkably, plasmids p5C-P121A and p5C-P/A led to full complementation of the Δ*efp* phenotype and even to higher CadA activities in wildtype cells containing plasmid p5C. Δ*efp* cells harboring plasmids p5C-P120A and p5C-P122A achieved only 21% and 47% of the wildtype β-galactosidase activities, respectively (Fig. 18C). As expected, wildtype CadC (p5C) and the P124A exchange failed to show any remarkable CadA activity in the Δ*efp* mutant.

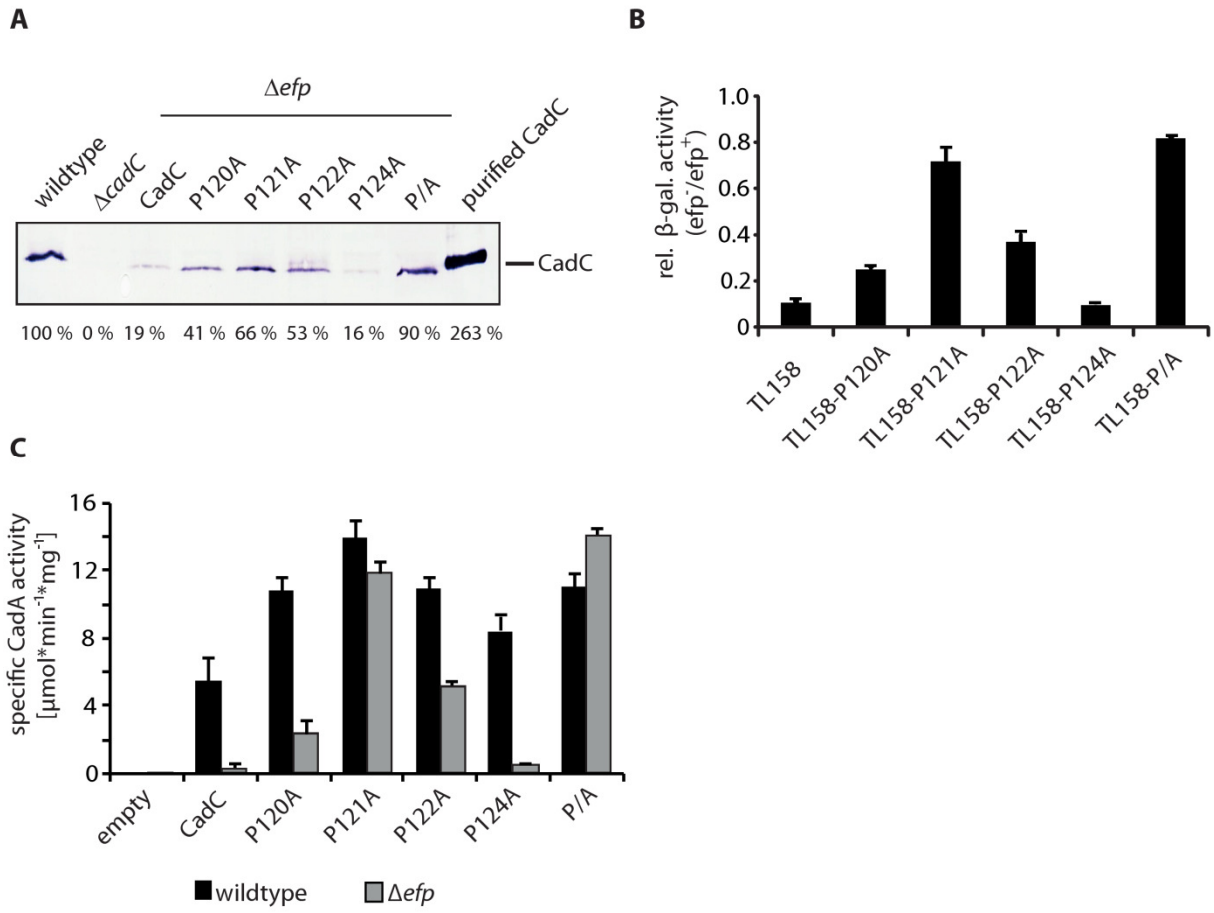


Figure 18: Disruption of the CadC proline cluster leads to EF-P independency.

A) CadC protein levels in various mutants. The protein levels of full-length CadC were determined by quantitative Western blotting using CadC-specific antibodies. A *cadC* deletion mutant was used as negative control, and wildtype CadC in the *efp*$^+$ strain was assigned to 100%. The CadC signal intensity was analyzed by using the program ImageJ.

B) β-Galactosidase activities in wildtype BW25113 (*efp*$^+$) and JW4107 (*efp*$^-$) cells harboring translational CadC´-LacZ fusions (TL158) and the corresponding proline/alanine CadC substitutes.

C) The CadA phenotype in in wildtype BW25113 (*efp*$^+$) and JW4107 (*efp*$^-$) cells harboring p5C (full-length *cadC*) and the corresponding proline/alanine CadC substitutes.

3.5.2. The specificity of the consecutive prolines

It could be shown above that *E. coli* CadC contains three consecutive proline residues, which are sufficient for ribosomal stalling and EF-P dependency. To determine if three prolines are generally effectual or if the number of prolines can vary, five *cadC'-lacZ* reporter fusions

were generated in plasmid p3LC encoding truncated $CadC_{1-30}$ (TL30) followed by one to five consecutive prolines and the LacZ reporter. The resulting plasmids p3LC-TL30-5Pro, p3LC-TL30-4Pro, p3LC-TL30-3Pro, p3LC-TL30-2Pro and p3LC-TL30-1Pro were expressed in wildtype BW25113 and JW4107 (Δ*efp*), repectively. Cells were cultivated in LB medium, harvested at exponential growth phase and the β-galactosidase activities were measured (Fig. 19A). When one or two prolines were inserted (TL30-1xPro and TL30-2xPro), the β-galactosidase activities were as high as in the control TL30-hybrid (no Pro) in efp^+ and efp^- cells. By contrast, β-galactosidase activities were highly reduced in the Δ*efp* mutant, when the *cadC'-lacZ* reporter fusions contained three or more consecutive prolines (TL30-3xPro, TL30-4xPro, TL30-5xPro). Moreover, the EF-P dependency seemed not to be enhanced with more than three prolines. Thus, three prolines in a row are sufficient for full EF-P dependent translation.

To define the amino acid specificity of EF-P mediated translation, several reporter fusions were constructed. Hence, a five amino acid stretch of each of the 20 proteinogenic amino acids was inserted after 30 aa of CadC (TL30) followed by a fusion to LacZ. After the strains BW25113 and JW4107 (Δ*efp*) harboring the plasmids were grown in LB medium and harvested at exponential growth phase, the β-galactosidase activities were measured. Strikingly, EF-P dependency was only visible in cells expressing the TL30-3xPro hybrid, but not in any of the other CadC´-LacZ reporters (Fig. 19B) indicating that proline is an exclusive amino acid for EF-P dependent translation.

Next, the role of proline codon usage for ribosomal stalling was examined. To rule out mRNA specific effects, four *cadC'-lacZ* reporter fusions were generated in plasmid p3LC encoding truncated $CadC_{1-30}$ (TL-30) followed by three consecutive prolines and the LacZ reporter. Repeats of the proline codons CCA, CCG or CCU were used to generate the constructs TL30-CCA, -CCG and -CCT, respectively, as well as a construct with the codon mixture CCA-CCA-CCT (TL30-Mix). β-Galactosidase activities were measured in strain BW25113 and JW4107 (Δ*efp*) for each of the four hybrids and were compared with the control TL30 that lacks the proline cluster (Fig. 20A). Translation of all four proline constructs was found to be impaired in strain JW4107 indicating that the amino acid proline in the nascent chain, rather than the type of proline codon in the mRNA, causes the EF-P dependency. This is consistent with the fact that the proline cluster of CadC is encoded by different proline codons, having the sequence CCU-CCC-CCU-ATA-CCA.

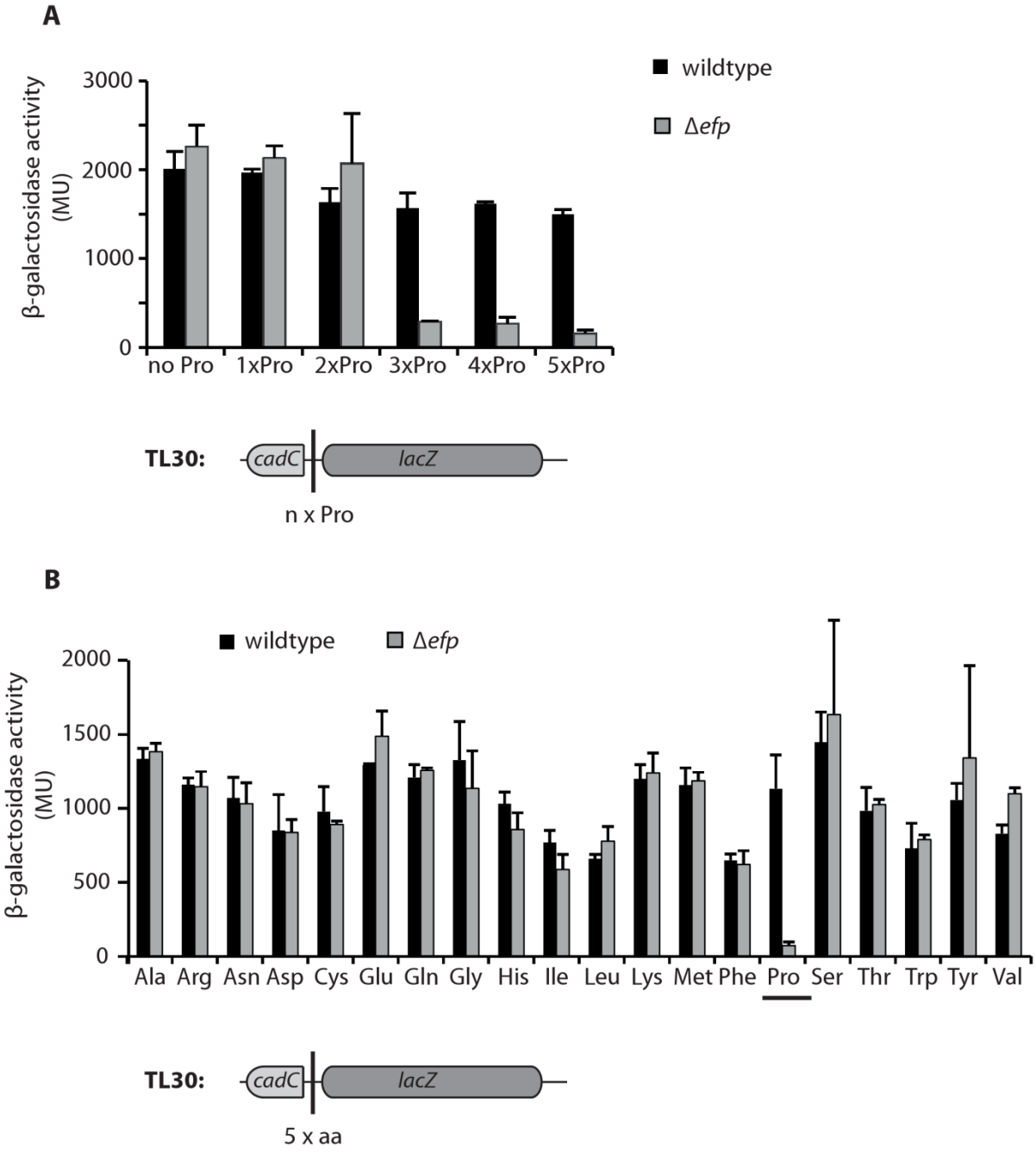


Figure 19: Three prolines are sufficient for ribosomal stalling.

A) The β-galactosidase activities of $CadC_{1-30}$-LacZ (TL30) hybrids bearing up to 5 prolines (see scheme) were measured in BW25113 wildtype and in JW4107 (Δ*efp*).

B) The β-galactosidase activities of $CadC_{1-30}$-LacZ (TL30) hybrids bearing five consecutive repeats of each of the 20 proteinogenic amino acids (see scheme) were measured in BW25113 wildtype and in JW4107 (Δ*efp*).

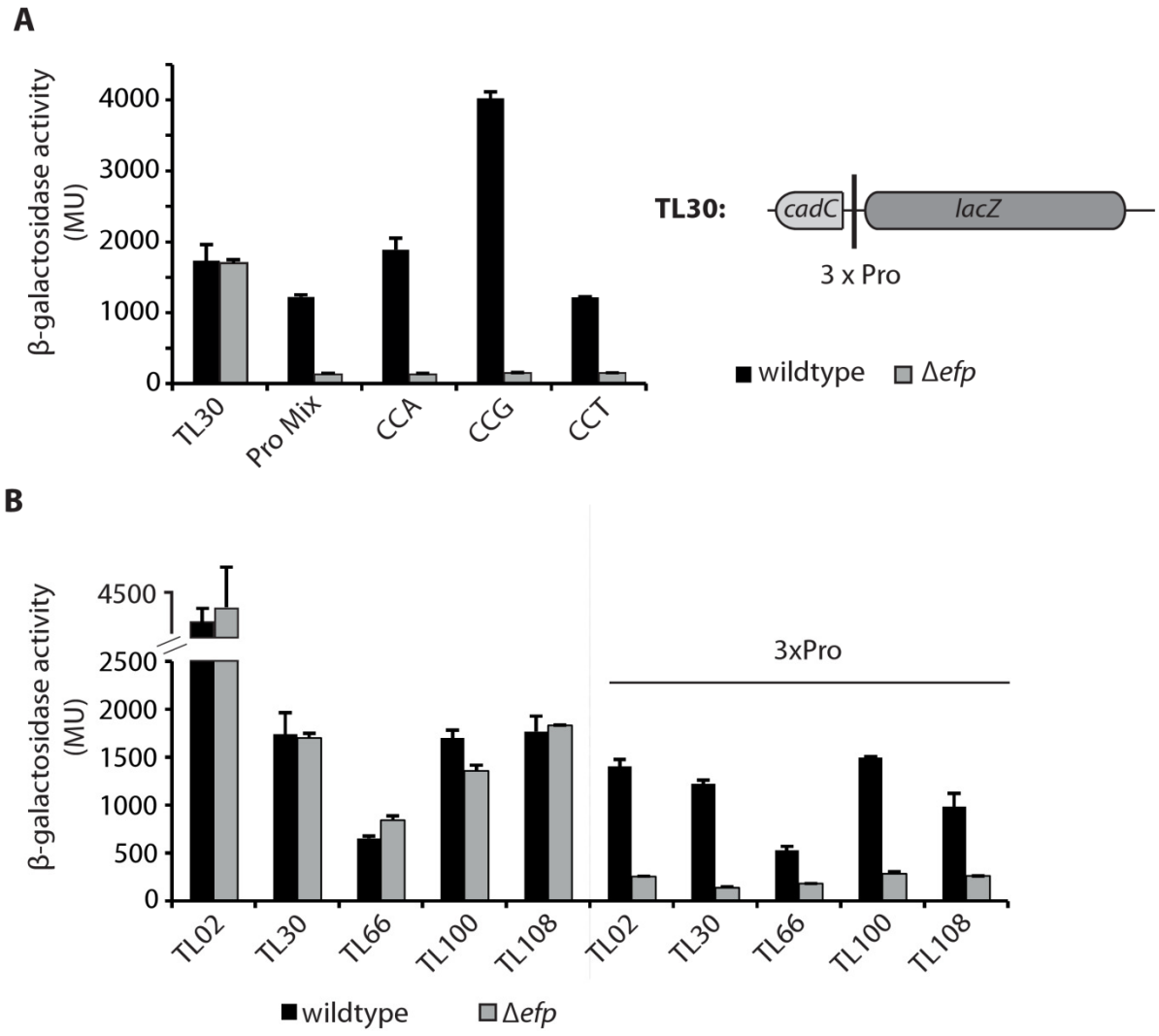


Figure 20: EF-P relieves stalling at polyproline-stretches.

A) Effect of codon variations of three consecutive prolines on EF-P mediated translation. The β-galactosidase activities of cells expressing the truncated CadC'-LacZ translational fusion TL30 with three consecutive prolines (3xPro) composed of different proline codons or a codon mixture were determined in BW25113 wildtype and JW4107 (Δ*efp*) cells.

B) Effect of the context of three consecutive prolines on EF-P mediated translation. Three prolines were positioned at the C-terminal end of truncated CadC' variants of various lengths fused to LacZ as depicted in Fig. A. The β-galactosidase activities of cells expressing these translational fusions with (3xPro) or without (no Pro) three consecutive prolines were determined in BW25113 wildtype and JW4107 (Δ*efp*) cells.

In a parallel approach it was examined whether the context of the cluster of prolines is important for EF-P dependent stalling. Five *cadC'-lacZ* reporter fusions encoding truncated

CadC of varying lengths, namely 2, 30, 66, 100 and 108 amino acids, followed by three consecutive prolines (3xPro) and LacZ were generated (TL02-3xPro, TL30-3xPro, TL66-3xPro, TL100-3xPro, TL108-3xPro). In addition, respective CadC´-LacZ constructs were designed lacking the 3xPro insertion. As expected, β-galactosidase activities of these control constructs were indistinguishable between strain BW25113 and JW4107 (Δ*efp*) (Fig. 20B, no Pro). By contrast, translation was strongly impaired for all 3xPro constructs in the Δ*efp* cells (3xPro) indicating that the context of the three prolines is of negligible importance for EF-P dependent translation. However, the strength of EF-P dependency varied (see 3.9.1).

3.5.3. Testing ribosomal stalling in vitro

To investigate whether the ribosome stalls at the position of the proline cluster of CadC during translation in the absence of EF-P, an in vitro transcription-translation system reconstituted from purified components was employed (Shimizu *et al.*, 2001). This work was done by Dr. Agata Starosta (Gene Center, Munich). Translation of wildtype CadC over a period of 5-30 min was monitored using incorporation of radiolabelled [^{35}S]-methionine into synthesized polypeptides (Fig. 21A, lanes 1-4). Synthesis of full-length CadC (FL at ~60 kDa) was observed after 10 min (lane 2) and increased with longer incubation (lanes 3-4). Interestingly, a prominent band of 35 kDa was observed at 5 min and persisted for 15 min (lanes 1-3), before becoming reduced or absent at 30 min (lane 4), consistent with a translation intermediate that eventually elongates to form the FL CadC product. Furthermore, this band was absent when the same reaction was performed in the presence of modified EF-P (Fig. 21A, lanes 5-8). The size of the 35 kDa product matches that of a CadC nascent polypeptide chain translated up to the proline cluster (~15 kDa), but still remaining attached to a tRNA (~20 kDa). The in vitro translation of CadC-PPPIP/AAAIS was also performed in the presence and absence of EF-P (Fig. 21B, lanes 9-14). In this case, the 35 kDa band was absent, and the presence or absence of EF-P had no influence on the reaction. As additional controls, in vitro translation was performed using CadC truncated directly after the proline cluster ($CadC_{1-125}$) revealing a product of the same size as in wildtype CadC (compare lanes 15 and 17 in Fig. 21B). Moreover, both the 10 min 35 kDa CadC intermediate (lane 15) and 35 kDa $CadC_{1-125}$ product (lane 17) could be reduced in size to 15 kDa via degradation of the tRNA by RNase A treatment (Fig. 21B, lanes 16 and 18). Collectively, these data suggest that the ribosome stalls at the proline cluster during CadC translation in the absence of EF-P and that the presence of EF-P alleviates the translational stalling.

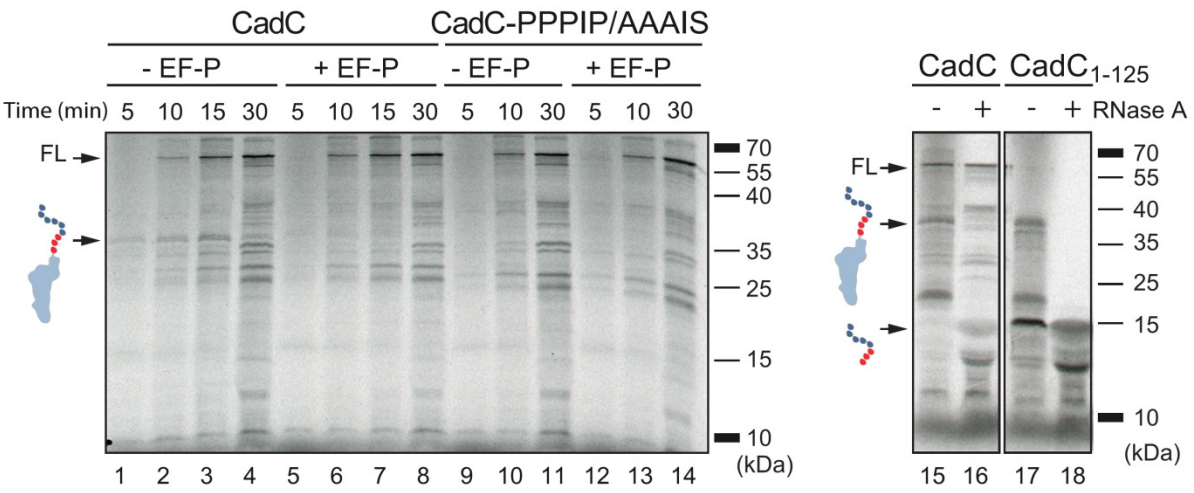


Figure 21: In vitro translation of CadC. Autoradiographs of SDS-PAGE analysis of [^{35}S]Met-labeled in vitro translation time courses of CadC and CadC-PPPIP/AAAIS, in the absence and presence of modified EF-P. Control reactions of CadC and truncated $CadC_{1-125}$ in the presence and absence of RNase A were performed. Positions of full-length CadC (FL), peptidyl-tRNA and free peptide are arrowed. This work was done by Dr. Agata Starosta (Gene Center, Munich).

3.6. EF-P and its physiological role in the Cad system

3.6.1. Comparison of *E. coli* and *V. harveyi* CadC

Enterobacterial and vibrional CadC proteins are conserved with respect to the domain architecture and share a high sequence homology comparing the HTH-DNA binding domain, though sequence similarity especially in the cytoplasmic region is low (Fig. 22A, B). Interestingly, the proline cluster present in *E. coli* is not found in *Vibrio* species (Fig. 22B). In addition, *Vibrio* lacks LysP. LysP acts as inhibitor of CadC and its overproduction leads to abolished *cadBA* expression even under inducing conditions (Neely *et al.*, 1994). This raises the hypothesis that EF-P might work as novel kind of translational regulator to control certain protein levels in *E. coli*. In the case of CadC this would mean that the level of CadC is crucial to overcome LysP expression and thus to induce *cadBA* expression under stress conditions.

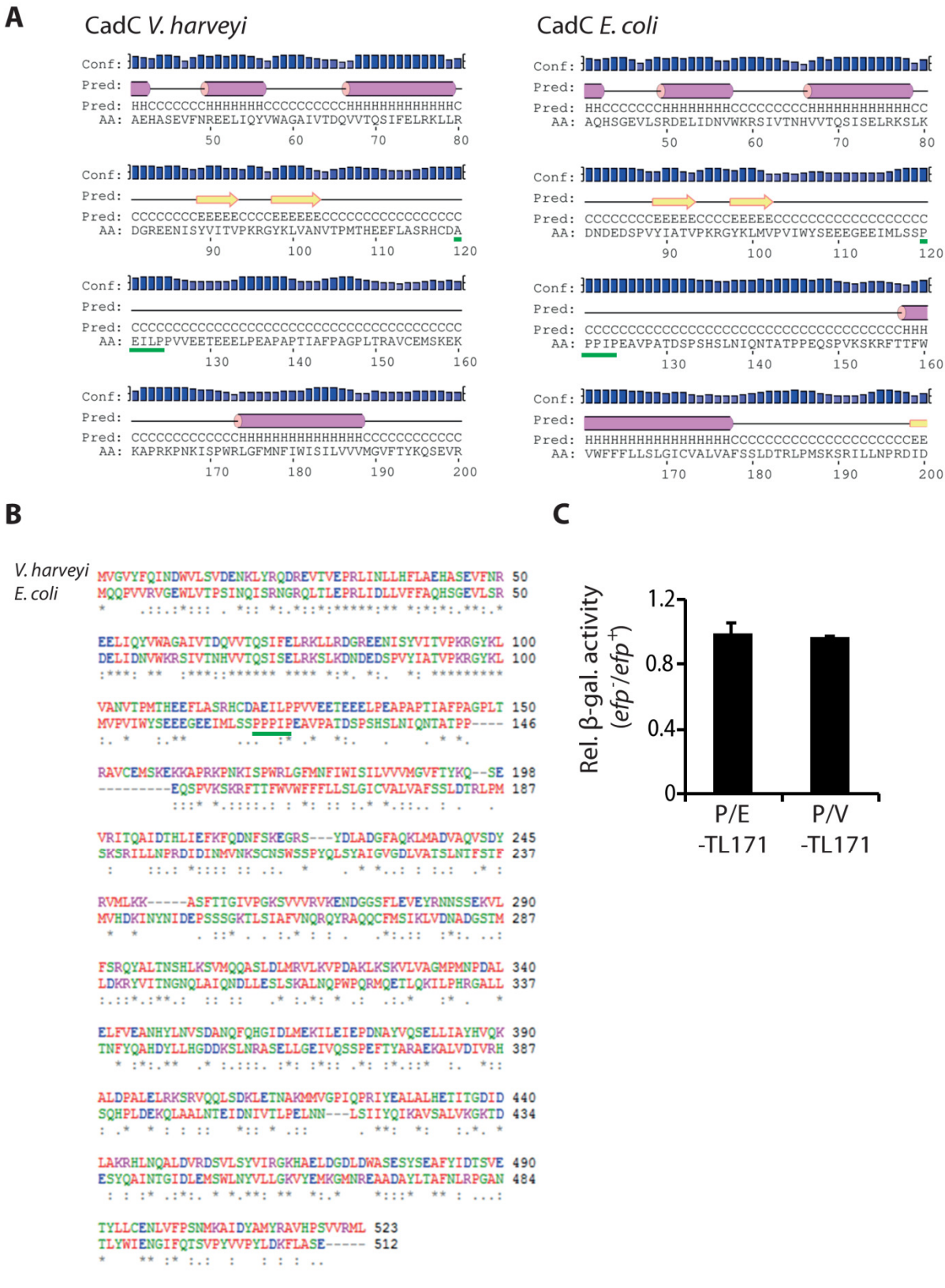


Fig. 22: Comparison of CadC in *V. harveyi* and *E. coli*.

A) Secondary structure prediction of *V. harveyi* and *E. coli* $CadC_{1\text{-}200}$ using PSIPRED.

B) Amino acid alignment of *V. harveyi* and *E. coli* CadC using Clustal Omega.

C) *Vibrio* CadC lacking the three consecutive prolines is EF-P indendent. Cells of wildtype BW25113 (*efp*$^+$) and JW4107 (*efp*$^-$) were grown in LB medium. Relative β-galactosidase activity is given as ratio *efp*$^-$/*efp*$^+$ values. P/E: promoter of *E. coli*. P/V: promoter of *V. harveyi*. 171 amino acids of Vibrio CadC were N-terminally fused to LacZ.

To test this hypothesis, the translational *cadC´-lacZ* fusion TL158 was employed replacing *E. coli cadC* by the orthologous 171 codon long counterpart of *Vibrio harveyi* (TL171). The gene was set either under the control of the *E. coli cadC* promoter or the *V. harveyi cadC* promoter. The resulting plasmids p3LC- P/E-TL171V (P/E stands for the *E. coli* promoter) and p3LC- P/V-TL171V (P/V stands for the *Vibrio* promoter) were expressed in wildtype BW25113 and in JW4107 (Δ*efp*) cells in LB medium. β-Galactosidase activities of the wildtype (*efp*$^+$) and the mutant (*efp*$^-$) were compared and indicated by an *efp*$^-$/*efp*$^+$ ratio. If EF-P would act as specific translational regulator, an EF-P independent phenotype is expected for *V. harveyi* CadC lacking the prolines. Indeed, cells harboring p3LC-P/E-TL171V and p3LC-P/V-TL171V revealed no reduced β-galactosidase activities when expressed in JW4107 (Fig. 22C). This result stands in contrast to the result obtained with *E. coli* CadC (Fig. 16B).

3.6.2. The consequence of EF-P independent CadC for *cadBA* expression

cadBA expression is regulated by the lysine dependent CadC inhibitor LysP, the small DNA binding protein H-NS and the transcription activator CadC. To define the regulatory role of EF-P in the Cad system, the influence of the EF-P independent CadC variant P/A on *cadBA* expression was further investigated. Cells of strain MG-CL-123 were transformed with plasmids p5C (wildtype CadC) and p5C-P/A (CadC-P/A). Cells were grown microaerobically in KE medium under inducing (pH 5.8 with 10 mM lysine) and non-inducing conditions (pH 5.8, pH 7.6 with or without 10 mM lysine) and harvested at their exponential growth phase. Then, β-galactosidase activities were measured. As mentioned before, activation of wildtype CadC requires the two stimuli exogenous lysine and low pH to induce *cadBA* expression. For strain MG-CL-123 harboring plasmid p5C-P/A, only one stimulus (either exogenous lysine or low pH) was sufficient for the induction of the *cadBA* operon (Fig 23A). In addition, partial induction was even observed without any stimulation.

To find out, if the unregulated *cadBA* expression in cells harboring plasmid p5C-P/A is related to an enhanced *cadC* translation, the amount of CadC was determined by Western Blot analysis using CadC specific antibodies.

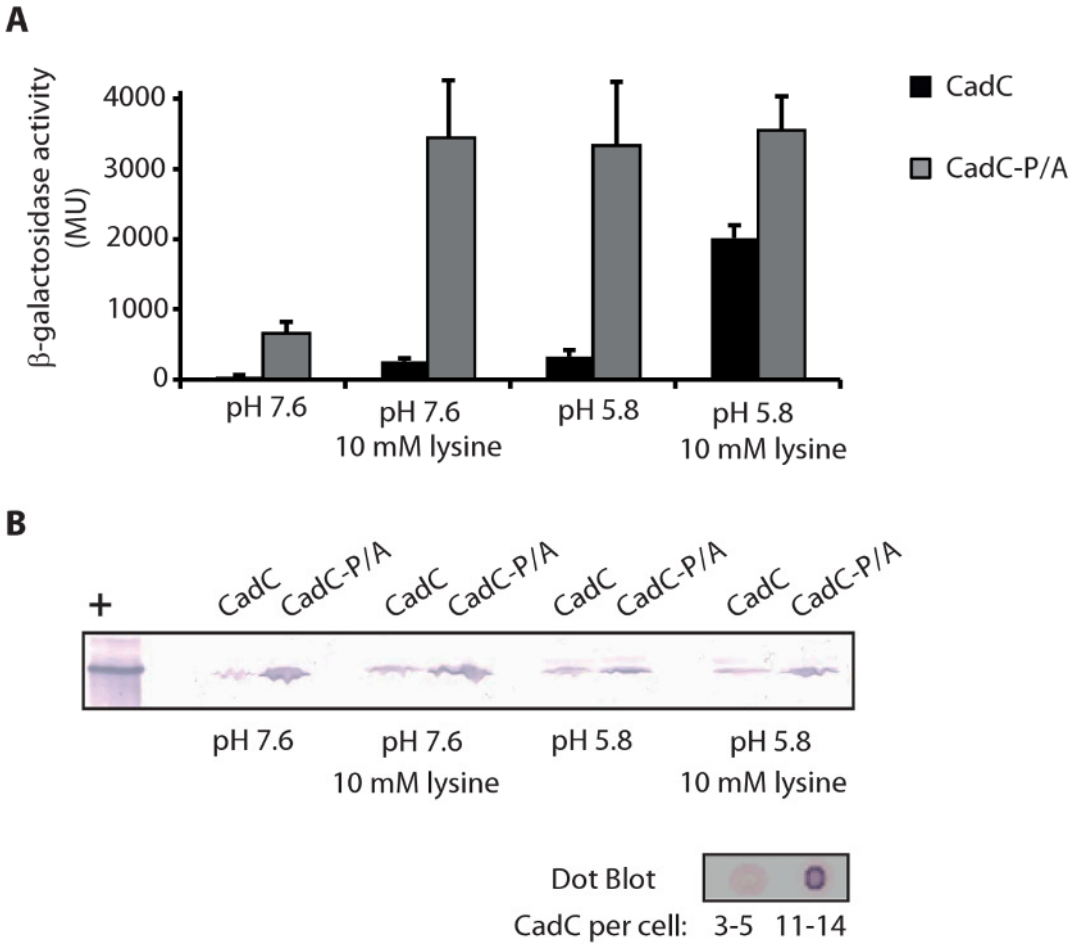


Figure 23: EF-P controls the tight copy number of CadC.

A) *cadBA* expression induced by wildtype CadC or the EF-P independent variant CadC-P/A. Cells of strain MG-CL-123 (MG1655 Δ*lacZ* Δ*cadBA* Δ*cadC* P_{cadBA}-*lacZ*) harboring plasmid p5C (CadC) or p5C-P/A (CadC-P/A), respectively, were cultivated microaerobically in KE minimal medium under non-inducing (pH 7.6 with or without 10 mM lysine, pH 5.8) and inducing conditions (pH 5.8 with 10 mM lysine).

B) Western Blot with antibodies against CadC. 10 µl of cells adjusted to OD_{600} = 10 was loaded per lane. The Dotblot was quantified with ImageJ. Cells were cultivated as described in Fig. 23A.

As expected, the amount of the CadC-P/A variant exceeded the wildtype CadC level under every condition. Additionally, levels of CadC were detected by Dotblot analysis using CadC-specific antibodies for cells grown under inducing conditions (pH 5.8 with 10 mM lysine). Wildtype CadC contained 3 - 5 molecules per cell, whereas the CadC-P/A variant contained 11 - 14 CadC molecules per cell, exceeding the number of wildtype CadC approximately

three times (Fig. 23B). Thus, EF-P mediated translation is crucial to maintain low CadC levels.

3.6.3. The effect of different CadC copy numbers on *cadBA* expression

To corroborate the correlation between the stimulus-dependent transcriptional activity and the copy number of CadC in more detail, wildtype CadC copy number was varied by expressing *cadC* from different plasmids. Therefore, cells of strain MG-CL-123 were transformed with plasmids pET16b-CadC, p5C and pBAD24-CadC, which allowed adjustment of low (3 - 5), medium (5 - 8) and high (23 - 28) CadC molecule numbers per cell, respectively. The different protein copy numbers were obtained by variations in promoter strength, RNA-polymerase dependency and plasmid copy number (Antoine & Locht, 1992; Cronan, 2006; Guzman *et al.*, 1995). Cells of strain MG-CR were used as wildtype control. Levels of CadC were detected from cells grown in KE minimal medium by Dotblot analysis using CadC-specific antibodies and quantified with the software ImageJ.

In CadC wildtype cells, the *cadBA* operon was only induced by both stimuli low pH and lysine. This is reflected by an increase of the CadC copy number of 1 - 3 molecules per cell at pH 7.6 to 3 - 4 molecules at pH 5.8 (Fig. 24A). This “CadC boost” can be mainly attributed to the derepression of *cadC* by H-NS at low pH leading to enhanced *cadC* transcript levels and thus to more CadC (Krin *et al.*, 2010). In addition, lysine is needed for *cadBA* induction, thus one stimulus (low pH) is not sufficient for *cadBA* expression. The presence of lysine weakens the inhibition of CadC by the lysine permease LysP (Tetsch *et al.*, 2008) and therefore, the *cad* operon is only expressed when two stimuli (exogenous lysine and low pH) are present. Cells expressing plasmid-encoded CadC at low protein level in strain MG-CL-123 harboring pET-16b-CadC showed only a slightly increased expression pattern in comparison to the wildtype (Fig. 24A). By contrast, an increase of CadC to a medium protein level (5 - 6 molecules per cell at pH 7.6, 7 - 8 molecules per cell at pH 5.8) in cells harboring plasmid p5C caused significant higher *cadBA* expression in response to either low pH or available lysine. Finally, a high level of CadC (23 - 25 molecules per cell at pH 7.6, 25 - 28 molecules per cell at pH 5.8) in cells harboring pBAD24-CadC led to stimulus-independent full induction of *cadBA* expression. These results clearly indicate the importance of a tight control of the CadC copy numbers for regulated *cadBA* expression and acid stress response.

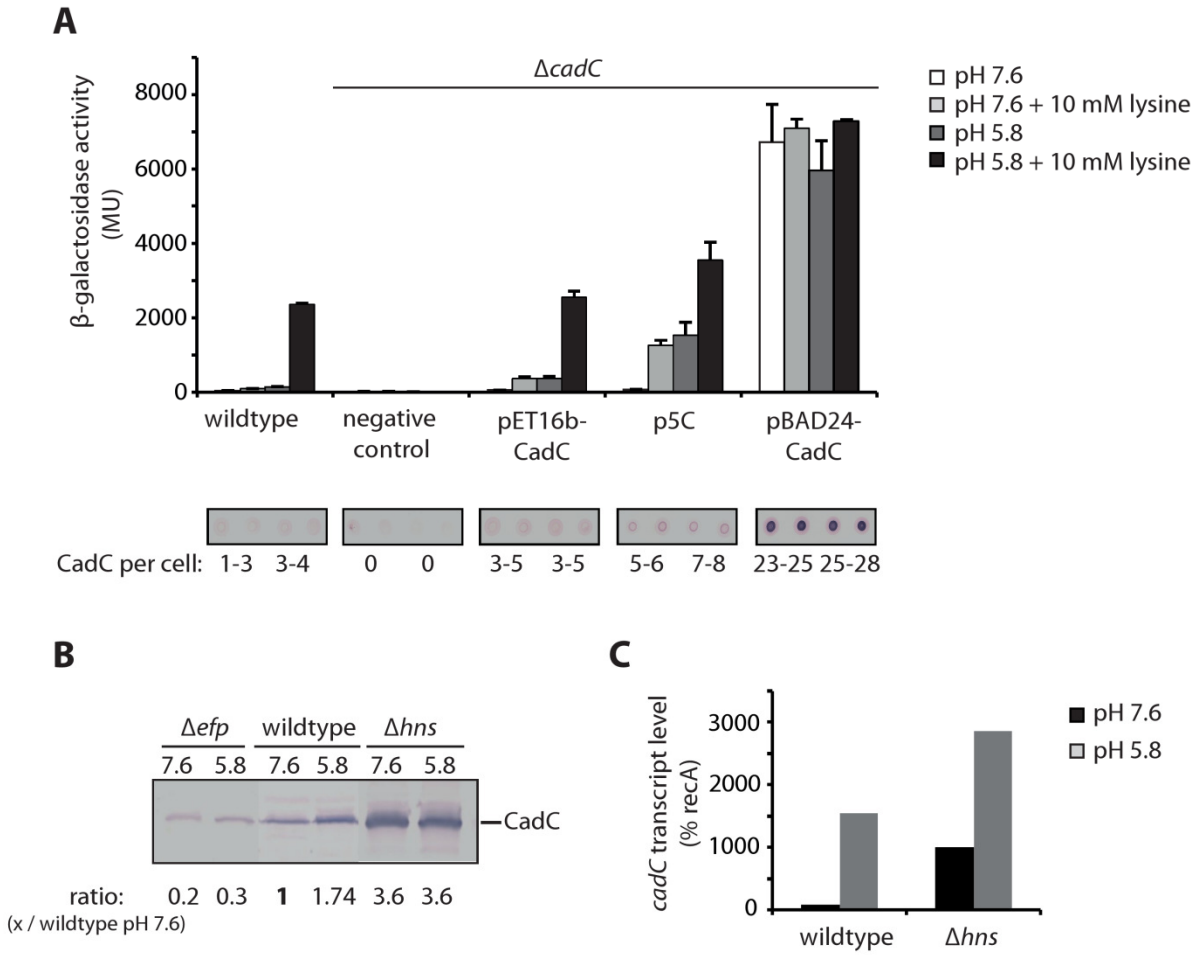


Figure 24: CadC amount is crucial for regulated stress response.

A) Influence of CadC copy number on *cadBA* expression. Strains MG-CR (*cadC* wildtype, P_{cadBA}-*lacZ*), MG-CL-123 (Δ*cadC,* P_{cadBA}-*lacZ*) and MG-CL-123 harboring plasmids pET16b-CadC (for low CadC amount), p5C (for medium CadC amount) and pBAD24-CadC (for high CadC amount) were grown under non-inducing (pH 7.6; pH 7.6 + 10 mM lysine; and pH 5.8) and inducing conditions (pH 5.8 + 10 mM lysine). Levels of CadC were detected by Dotblot analysis using CadC-specific antibodies and quantified with the software ImageJ.

B) Comparison of CadC levels in cells grown under non- and inducing conditions. Cells of BW25113 wildtype, JW1225 (Δ*hns*) and JW4107 (Δ*efp*) harboring plasmid p5C were cultivated under non-inducing conditons (pH 7.6) in KE minimal medium and then shifted to inducing conditions (pH 5.8, 10 mM lysine) for 1 h. 150 µg membrane vesicles were loaded per lane. The Western Blot analysis was performed using CadC specific antibodies. Quantifications were made with the software ImageJ.

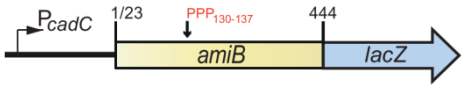
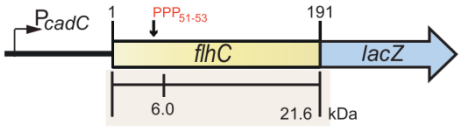
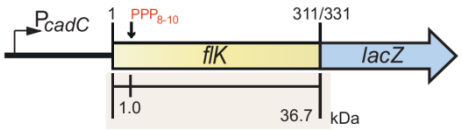
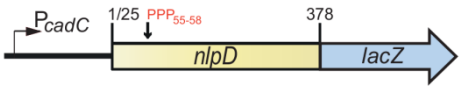
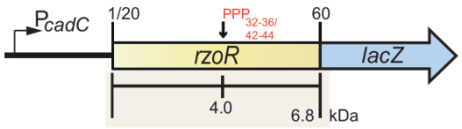
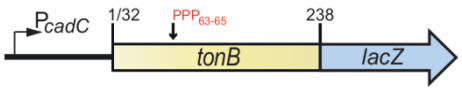
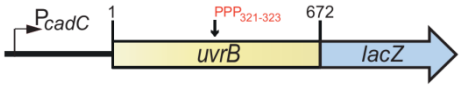
C) *cadC* transcript levels in the BW25113 wildtype and the JW1225 (Δ*hns*) mutant. Transcript levels were determined by qRT-PCR. Cells were cultivated as described in Fig. 24B.

The “CadC boost” at pH 5.8 could be also detected for cells grown in KE minimal medium by Western Blot analysis using membrane vesicles of BW25113 harboring plasmid p5C and CadC specific antibodies (Fig. 24B). For the Western Blot, cells of BW25113 wildtype and JW4107 (Δ*efp*) harboring p5C were cultivated under non-inducing conditions (pH 7.6 without lysine) and were then shifted to lysine-rich low pH medium. Indeed, membrane vesicles of wildtype cells, which were shifted to inducing conditions, contained 1.74 times more CadC protein than those from cells grown under non-inducing conditions (Fig. 24B).
Additionally, cells of strain JW1225 (Δ*hns*) harboring plasmid p5C were cultivated like the wildtype above to confirm the correlation between the CadC amount and H-NS dependent regulation. As expected, Δ*hns* cells revealed 3.6 times higher CadC amounts under non- and under inducing conditions in comparison to the wildtype (pH 7.6). This result could be confirmed by qRT-PCR for cells grown in LB medium (Fig. 24C) standing in good accordance to the results of Krin *et al.* (Krin *et al.*, 2010).

3.7. Identification of additional EF-P dependent proteins

The proteome of *E. coli* contains approximately 100 proteins with three or more prolines, including six proteins with four (EntS, YcgL, Cls, YdeI, MtlR, YodB), two with five (RzoR, YaaX) and one with eight consecutive prolines (AmiB) (Table A1). To test whether these proteins are also EF-P dependent, eight proteins were chosen, which have the proline cluster in the first half of their coding sequence. These proteins - AmiB, FlhC, Flk, HisD, NlpD, RzoR, TonB and UvrB - differ in function, cellular localization, proline number and location of the polyproline stretch (Table 6). Expression of the corresponding genes was set under control of the *cadC* promoter to ensure low and equal transcriptional levels, and genes were fused to *lacZ* in plasmid p3LC. In the case of *rzoR*, *tonB*, *amiB* and *flk*, the sequence encoding the signal peptide or the periplasmic domain, respectively, had to be removed since LacZ is only active in the cytoplasm. Strains BW25113 and JW4107 (Δ*efp*) were transformed with the resulting plasmids. After cultivation in LB medium, cells were harvested at the exponential growth phase and the β-galactosidase activities were measured. Except of HisD-LacZ, all other hybrids were EF-P dependent and revealed lower β-galactosidase activities in the Δ*efp* mutant as in the wildtype (Fig. 25A).

Table 6: EF-P dependency of various polyproline-containing proteins in *E. coli*. Translational gene-*lacZ* fusions were placed under the control of the *cadC* promoter to ensure low and equal transcriptional levels. In case of *amiB*, *flk*, *nlpD*, *rzoR* and *tonB*, the sequence encoding the signal peptide or the transmembrane domain (*flk*) were removed since LacZ is only active in the cytoplasm. Positions of the corresponding first and last amino acids as well as of the proline stretches are marked by numbers. Full length and truncated sizes of the gene products tested in in vitro transcription/translation assays (Fig. 25) are marked below each gene.

Protein	**Function**	**Protein localization**	**Proline number**[a]	**Schematic of the translational *lacZ* fusions**[b]
AmiB	N-acetylmuramoyl-l-alanine amidase	periplasm	8	
FlhC	transcription activator of flagellar genes	cytoplasm	3	
Flk	predicted flagella assembly protein	cytoplasmic membrane	3	
HisD	histidinol dehydrogenase	cytoplasm	3	
NlpD	predicted lipoprotein	outer membrane	3	
RzoR	predicted lipoprotein	outer membrane	5 and 3	
TonB	energy transducer	cytoplasmic membrane	3	
UvrB	excision nuclease subunit B	cytoplasm	3	

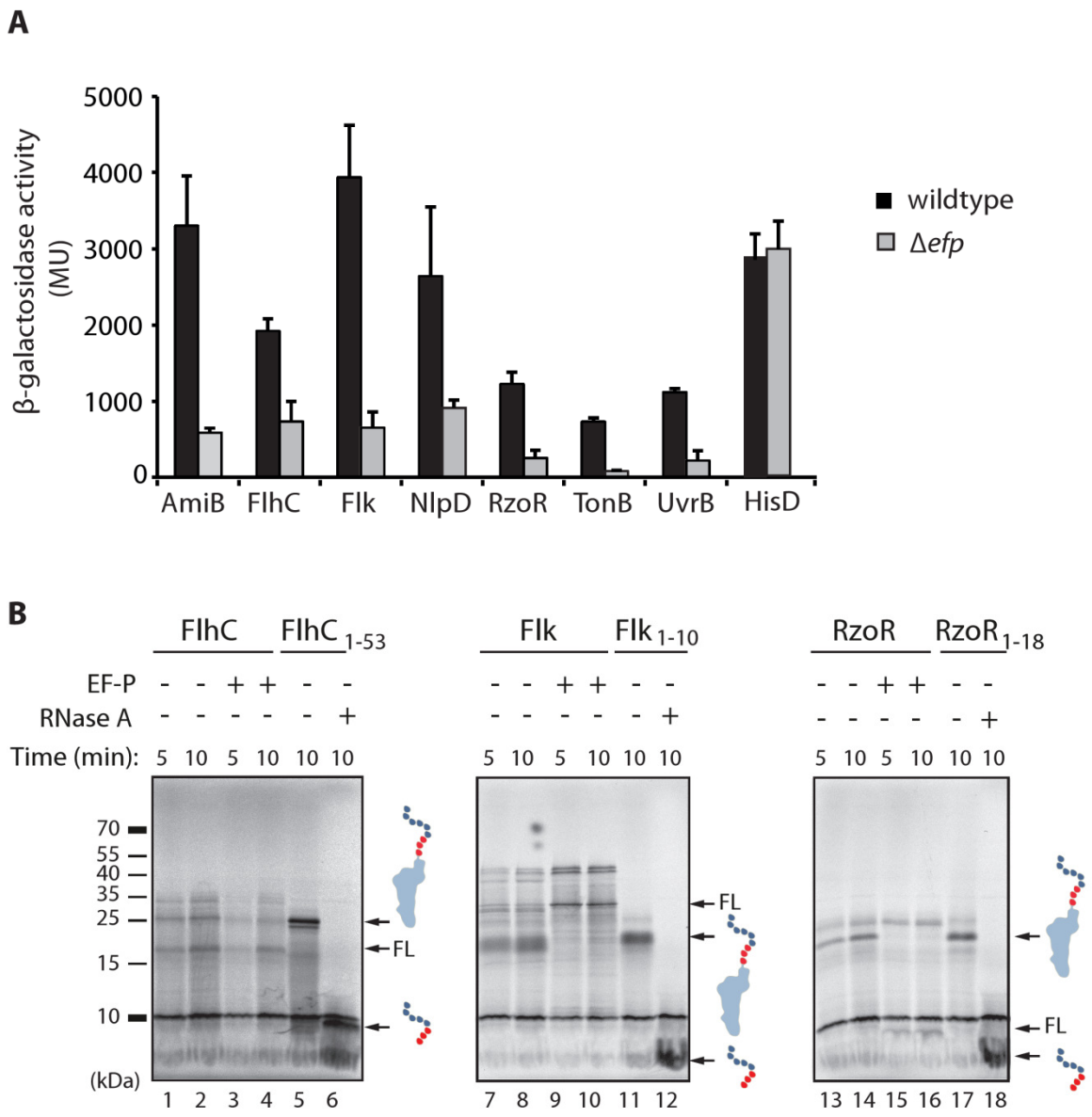


Fig. 25: EF-P dependent proteins.

A) The β-galactosidase activities of translation fusions of the polyproline-containing *E. coli* proteins AmiB, FlhC, Flk, NlpD, RzoR, TonB and UvrB with LacZ were determined in BW25113 wildtype and JW4107 (Δ*efp*).

B) Autoradiographs of SDS-PAGE analysis of [^{35}S]Met-labeled in vitro translation time courses of FlhC, Flk and RzoR performed in the absence and presence of modified EF-P. Control reactions with truncated $FlhC_{1-53}$, Flk_{1-10} and $RzoR_{1-18}$, with post-reaction RNase A treatment were performed. The position of full-length protein (FL), peptidyl-tRNA and free peptides are arrowed. This work was done by Dr. Agata Starosta (Gene Center, Munich).

To determine whether the proline clusters in these diverse proteins also cause translational stalling that can be alleviated by EF-P, in vitro translation assays were employed using *flhC*, *flk* and *rzoR* as templates (Fig. 25B). This work was done by Dr. Agata Starosta (Gene Center, Munich). The expected size of the full-length FlhC is 21.6 kDa and stalling at $_{51}$PPP$_{53}$ would produce a 6 kDa nascent chain. Consistently, in the absence of EF-P, a band was observed at 26 kDa (lanes 1-2). This band corresponded to the FlhC$_{1-53}$ peptidyl-tRNA (lane 5), was sensitive to RNase A (lane 6) and became weaker in the presence of EF-P (lanes 3-4). In contrast to FlhC, the size of Flk is 36.7 kDa. It has a proline cluster, $_{8}$PPP$_{10}$, very close to the NH_2-terminus and stalling would produce a small 1 kDa nascent chain. Indeed, translation of Flk in the absence of EF-P led to the appearance of bands at ~21 kDa (lanes 7 - 8), which corresponded to the size of the truncated Flk$_{1-10}$ peptidyl-tRNA (lane 11). These bands were sensitive to RNase A (lane 12) and became significantly weaker in the presence of EF-P (lanes 9 - 10). Finally, the translation of RzoR was monitored, which is a small 4.7 kDa protein with five consecutive prolines, $_{14}$PPPPP$_{18}$. Stalling at this site would produce a 1.9 kDa nascent chain that would be visible by a 21.9 kDa peptidyl-tRNA product. In the absence of EF-P, a strong band was observed at ~22 kDa (lanes 13 - 14), which corresponded to the RzoR$_{1-18}$ peptidyl-tRNA (lane 17). The band was sensitive to RNase A (lane 18) and was not detected in the presence of EF-P (lane 15 - 16).

3.9 Proline cluster and the “plus”

In a next approach it was investigated if the proline stretch is sufficient for fully EF-P dependency, or if other factors – like the proline localization or other amino acids – could influence EF-P related ribosomal stalling.

3.9.1 Importance of proline localization

The locations of the proline clusters of the eight tested proline rich proteins were analyzed with respect to the predicted secondary structures via PSIPRED (Buchan *et al.*, 2010). As shown in Fig. 26A, the proline clusters are located in unstructured regions varying in length and lead to EF-P dependency in a range from 60% (FlhC) to 90% (RzoR and TonB).

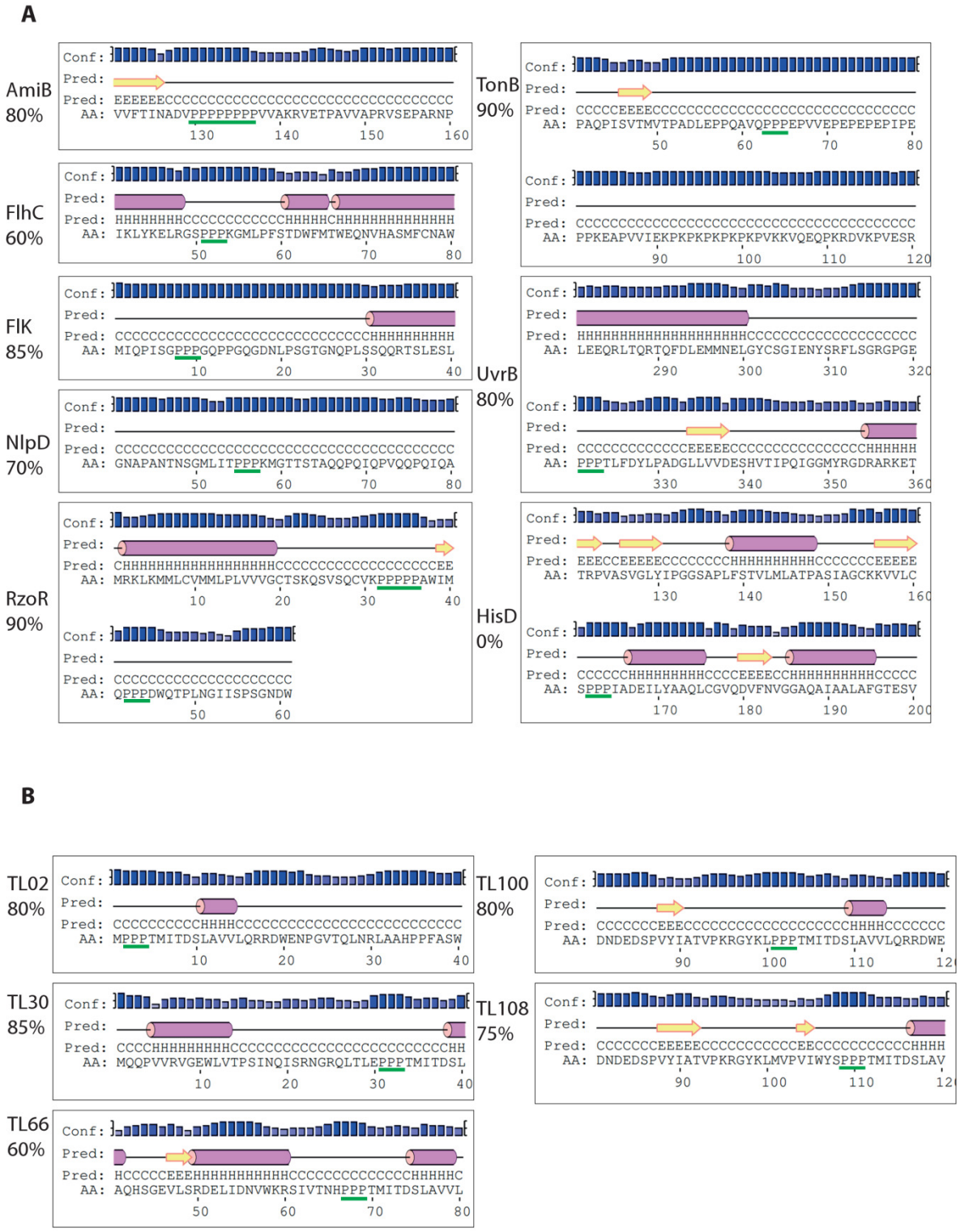


Figure 26: Secondary structures of regions containing proline clusters of proline-rich proteins (A) and CadC´-LacZ hybrids (3xPro) (B, see also Fig. 20B). Predictions were performed computer based using the program PSIPRED. EF-P dependency is given in %.

In CadC, which shows about 85% EF-P dependency (TL158, Fig. 16B), the proline cluster can be found in a long unstructured region consisting of more than 50 amino acids (Fig. 22B). In FlhC, revealing almost the lowest EF-P dependency of the tested proteins in vivo, the unstructured region consists of only 12 amino acids. The proline cluster is located two amino acids after an α-helix. Interestingly in the EF-P independent HisD, the proline cluster lays in an even shorter unstructured region, directly between an α-helix and a β-strand.
Considering again Fig. 20B (3xPro), the introduction of three consecutive prolines at various positions within CadC resulted in EF-P dependency in every variant, but the strength varied also in this experiment with the proline positions (Fig. 26B). In TL-66-PPP, the proline cluster is located in a relatively short unstructured region leading to 60% EF-P dependency. This result is comparable to the data obtained with FlhC. In TL-30-PPP and TL-100-PPP, the unstructured region harboring the proline cluster is longer than in TL-66-PPP, and also the EF-P dependency is higher (85% and 80%, respectively) in these two variants. Strains transformed with plasmid TL-108-PPP revealed 75% EF-P dependency. In the case of Flk and TL-02-PPP, the proline cluster can be found at the protein NH_2-terminus leading to strong EF-P dependency (85% and 80%, respectively).
Taken together, these results indicate that the secondary structure of the nascent polypeptide chain most likely plays an additional role in EF-P mediated translation elongation. Thus, the translation of proteins becomes preferentially EF-P dependent, when the proline cluster is located in a long unstructured region between two helices or if the cluster occurs directly after the start codon.

3.9.2 Prolines in combination with other amino acids

Next, it was tested if an exchange of the prolines against alanines in FlhC, which showed 60% EF-P dependency, could influence translation in the same way as in CadC. For that reason, three additional *flhC-lacZ* reporter fusions were constructed resulting in plasmids p3LC-FlhC-APP, p3LC-FlhC-PAP and p3LC-FlhC-AAA. Plasmids were transformed into strains BW25113 and JW4107 (Δ*efp*), cells were grown in LB medium and the β-galactosidase activities were measured. Comparable to the results obtained with CadC and the proline/alanine substitutes (Fig. 18), *efp* mutants harboring plasmid p3LC-FlhC-AAA with an exchange of all three prolines against alanines were less EF-P dependent as with wildtype FlhC and revealed 20% higher β-galactosidase activities (Fig. 27A). However, plasmid p3LC-FlhC-PAP did not lead to enhanced activities in the absence of *efp*, which was observed in

Δ*efp* cells harboring the CadC-PAP variant. Interestingly, plasmid p3LC-FlhC-APP led only to 10% of the wildtype activity. This raised the question, whether amino acids surrounding the proline cluster could also play a role in EF-P dependent translation. To investigate this aspect further, the amino acids upstream of the proline cluster of CadC, HisD and FlhD were observed in more detail. In CadC wildtype, two serines can be found next to the proline stretch (SSPPP). The EF-P independent HisD protein contains a cysteine and a serine next to the three prolines (CSPPP), and FlhC harbors a glycine and a serine (GSPPP). To test, if the HisD-CSPPP or the FlhD-GSPPP sequence could change the EF-P dependency in CadC, the sequences were exchanged in plasmid TL158, in which 158 amino acids of CadC are fused to the reporter LacZ. Cells of wildtype BW25113 and JW4107 (Δ*efp*) were transformed with the resulting plasmids TL158-CSPPP, TL158-GSPPP and TL158 as control. After cultivation in LB medium, the cells were harvested at exponential growth phase and the β-galactosidase activities between efp^+ and efp^- cells were compared and given as ratio efp^-/efp^+. In accordance to the former results, cells harboring wildtype CadC in TL158 revealed a ratio of 0.15. By contrast, plasmids TL158-GSPPP or TL158-CSPPP led to a ratio of 0.2 and 0.3, respectively (Fig. 27B), indicating that besides the secondary structure, amino acids upstream of the proline cluster can also lead to variations in the strength of EF-P dependency.

Finally, it was investigated if EF-P also relieves stalling at additional proline rich sequences different to PPP. It was demonstrated before that the exchange of a proline against alanine in the CadC variants CadC-APP and CadC-PPA could weaken the EF-P dependency, but the variants still showed more than 60% reduced β-galactosidase activities in comparison to *efp* wildtype cells (Fig. 18B). Plasmid p3LC-FlhC-APP resulted in even lower β-galactosidase activities in the *efp* mutant as with wildtype FlhC (Fig. 27A). This raises the question, if an amino acid other than proline could cause EF-P dependent stalling, when it is embebbed in the context of two prolines. To this end, *cadC*'-*lacZ* reporter fusions were generated encoding truncated $CadC_{1-30}$ followed by GPP, PGP or PPG, respectively, and the LacZ reporter (TL30-GPP, TL30-PGP, TL30-PPG). Strains BW25113 and JW4107 (Δ*efp*) were transformed with the resulting plasmids and with plasmids TL30 (no prolines) and TL30-PPP (with 3 prolines) as controls. After cultivation in LB medium, cells were harvested at exponential growth phase and β-galactosidase activities were measured. As shown before, strains harboring TL30-PPP showed a very high reduction of about 85% in β-galactosidase activity in the *efp* mutant compared to the wildtype (Fig. 27C). Remarkably, strains harboring TL-GPP and TL-PPG revealed EF-P dependency, but showed only a reduction of about 75% in the mutants standing

in good accordance with the results obtained with the CadC-APP and CadC-PPA variants mentioned before. Disruption of the proline stretch by a glycine in TL30-PGP led to about 50% EF-P dependency. These results confirm that besides three consecutive prolines also other proline-rich sequences can lead to EF-P dependent translation.

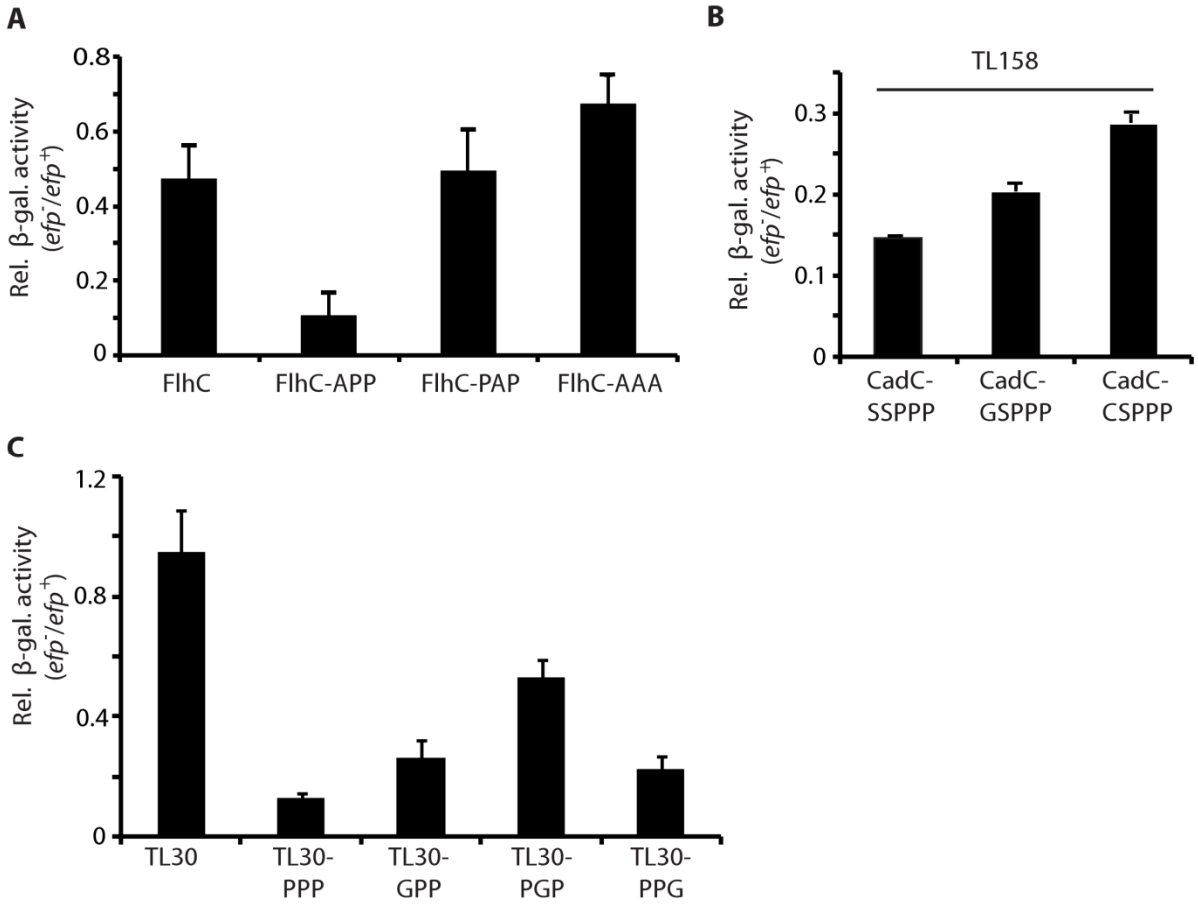


Figure 27: EF-P dependent translation of proteins containing proline-rich sequences.

A) Proline/alanine substitutions in FlhC. β-Galactosidase activities of cells of wildtype BW25113 and JW4107 (Δ*efp*) harboring p3LC-FlhC, p3LC-FlhC-APP, p3LC-FlhC-PAP and p3LC-FlhC-AAA are indicated by a ratio efp^-/efp^+.

B) Amino acid substitution upstream of the CadC proline cluster. β-Galactosidase activities of cells of wildtype BW25113 and JW4107 (Δ*efp*) harboring p3LC-TL158 (CadC wildtype: SSPPP), p3LC-TL158-GSPPP and p3LC-TL158-CSPPP are indicated by a ratio efp^-/efp^+.

C) Proline/glycine substitutions in CadC. β-Galactosidase activities of cells of wildtype BW25113 and JW4107 (Δ*efp*) harboring $CadC_{1\text{-}30}$ -LacZ (TL30) variants are indicated by a ratio efp^-/efp^+.

3.10 Differences in *cadBA* expression in KE and LB medium

The Δ*efp* mutant revealed abolished CadA activity in KE (Fig. 6B) as well as in LB medium (Fig. 29A). Cells of strains MG-CR-*yjeA* and MG-CR-*yjeK* cultivated in KE medium also showed highly reduced *cadBA* expression (Fig. 10). However in low pH LB medium, the *cadBA* operon was still expressed in the mutants and showed 28% and even 81% of the wildtype expression in the Δ*yjeK* strain and in the Δ*yjeA* strain, respectively (Fig. 28A). In addition, also strain MG-CR revealed at least two times higher β-galactosidase activities in LB medium than in KE medium (data not shown). To investigate possible reasons for this discrepancy, following experiments were conducted:

In Western Blot analysis, the CadC amounts were usually higher in cells grown in LB medium than in cells grown in KE medium (Fig. 28B). Therefore, it was investigated if *cadC* is transcribed more efficiently in LB medium than in KE medium. A higher *cadC* transcript amount could then result in higher *cadC* translation rate and to *cadBA* expression even in the absence of active EF-P. To further analyze this point, a qRT-PCR was performed testing *cadC* expression in LB medium and comparing the result with the *cadC* expression in KE medium. The *cadC* transcript level seems to be higher in LB medium than in KE medium, but the LB samples contained a very high standard deviation (Fig. 28C). Thus, *cadC* transcript levels probably vary only slightly in KE and in LB medium. However, these slight variations could be sufficient for higher *cadC* expression in complex medium, as only two more CadC molecules are enough for *cadBA* expression under inducing conditions in comparison to non-inducing conditions (see Fig. 24A).

Additionally, the *hns* and *efp* double mutant SU4 was constructed to investigate if an enhanced *cadC* transcript level can lead to an EF-P independent CadA phenotype. It was shown before that an *hns* mutant reveals enhanced *cadC* transcript levels and higher CadC amounts in comparison to the wildtype (Krin *et al.*, 2010; Fig. 24B, C). Therefore, the double mutant, wildtype BW25113 and the Δ*hns* and Δ*efp* single mutants as positive and negative controls, respectively, were incubated in inducing KE medium (pH 5.8 with 10 mM lysine), and CadA activities were determined. As expected, the Δ*efp* strain revealed no CadA activity, whereas the Δ*hns* strain exhibited a 40% higher CadA activity than the wildtype, reflecting the derepression of both the *cadC* and *cadBA* promoter (Fig. 28D). Remarkably, the double mutant also showed 30% more CadA activity than the wildtype. Previous studies have revealed that a double mutation in *hns* and *cadC* leads to a CadA negative phenotype comparable to *efp*$^-$ cells (Küper & Jung, 2005). This indicates that the expression of the

cadBA operon is exclusively induced by CadC, even when H-NS as repressor of the *cadBA* operon is missing. Therefore, the high CadA activity in the Δ*hns* Δ*efp* double mutant led to the assumption that a high transcript level of a gene can mask EF-P dependent ribosomal stalling.

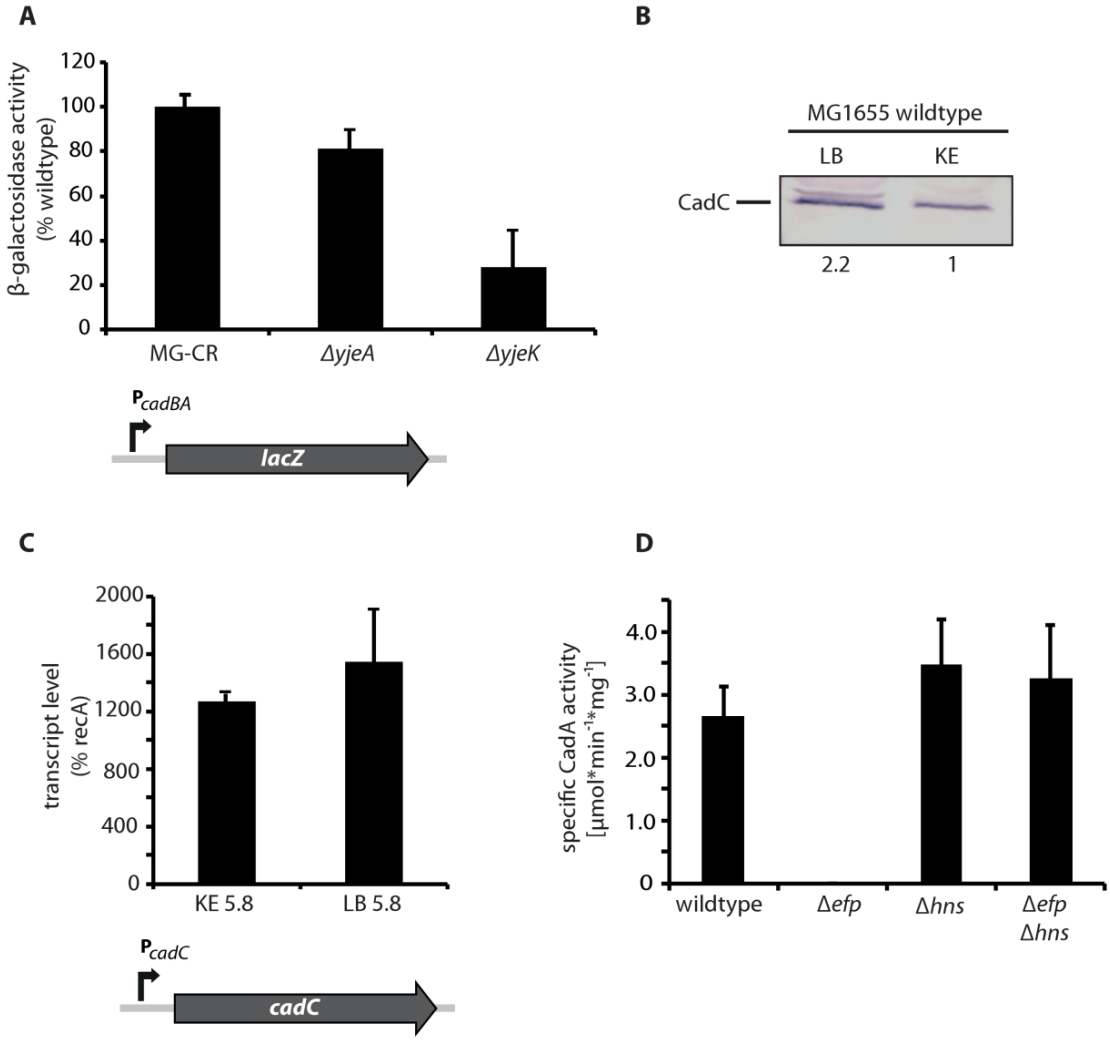


Fig. 28: Differences in *cadBA* expression in LB and KE medium.

A) β-Galactosidase activities of cells of MG-CR (P_{cadBA}-*lacZ*), MG-CR-*yjeA* (P_{cadBA}-*lacZ* Δ*yjeA*) and MG-CR-*yjeK* (P_{cadBA}-*lacZ* Δ*yjeK*) grown in LB medium.

B) CadC amount in MG1655 wildtype cells grown in LB and KE medium. Western Blot analysis was done with CadC-specific antibodies. Quantifications were made by using the software ImageJ.

C) *cadC* transcript levels in the BW25113 wildtype grown in KE minimal (pH 5.8, 10 mM lysine) or LB medium (pH 5.8). Transcript levels were determined by qRT-PCR.

D) CadA phenotype of JW4106 (Δ*hns*) mutant and SU4 (Δ*hns* Δ*efp*). Cells were grown under inducing conditions in KE minimal medium (pH 5.8, 10 mM lysine).

It is well known that LB is rich in nutrients, thus cells can grow faster and harbor more ribosomes than in minimal medium (Tao *et al.*, 1999). Therefore, it was tested if the amount of EF-P is also higher in LB than in KE medium resulting in higher CadC amounts in the complex medium. To test this, BW25113 wildtype cells were cultivated in LB and KE medium under inducing or non-inducing conditions to an OD_{600} of about 0.5. Subsequently, cells were harvested and adjusted to an OD_{600} of 10. 10 µl of each sample was loaded onto a 12.5% SDS gel, and a Western Blot was performed using EF-P specific antibodies. Indeed, the amount of EF-P was two times higher in LB medium than in KE medium, whereas no difference between cells grown in pH 7.6 or pH 5.8 could be observed (Fig. 29A). Thus, it is possible that a higher level of EF-P together with a higher level of ribosomes in LB is responsible for *cadC* translation and thus *cadBA* expression even in the absence of one of the EF-P modifiers (see Fig. 28A).

Interestingly, Keio strain JW4106 harboring a start to stop deletion of *yjeK* showed a partial rescue of the wildtype CadA activity even in KE minimal medium (Fig. 29B). As the start codon of *yjeK* can be found only 41 bp upstream of the translational start site of *efp*, strain JW4106 simultaneously harbors a shortened *efp* promoter. Thus, the CadA activity in this strain could be explained by a repressor binding site within the *efp* promoter region. A deletion of this site would then lead to enhanced expression of *efp* and to *cadBA* expression also in the *yjeK* mutant. As the modification of EF-P must be incomplete due to the lack of *yjeK* (Yanagisawa *et al.*, 2010), the wildtype CadA activity can be restored by only 77%, but one can speculate that an enhanced amount of α-lysylated EF-P could be sufficient to turn on the Cad system. To this end, strains BW25113, JW4107 (Δ*efp*), JW4106 (Δ*yjeK*), SU1 (Δ$yjeK_{643\text{-}1029}$) and JW1225 (Δ*hns*) were cultivated microaerobically in KE medium under inducing conditions until the cells reached an OD_{600} of about 0.5. Subsequently, cells were harvested, loaded onto a SDS gel, and a Western Blot was performed using EF-P specific antibodies. Indeed, in Keio strain JW4106 (Δ*yjeK*) the EF-P amount was 30% higher than in the wildtype (Fig. 29C). However, H-NS can be excluded as possible repressor, since an *hns* deletion did not result in enhanced EF-P levels. In addition, it was excluded that an enhanced amount of unmodified EF-P could lead to activation of the *cadBA* operon, since an induction of plasmid pQE70-Eco_*efp* K34A by IPTG could not complement the negative CadA phenotype in Δ*efp* cells (Fig. 29B).

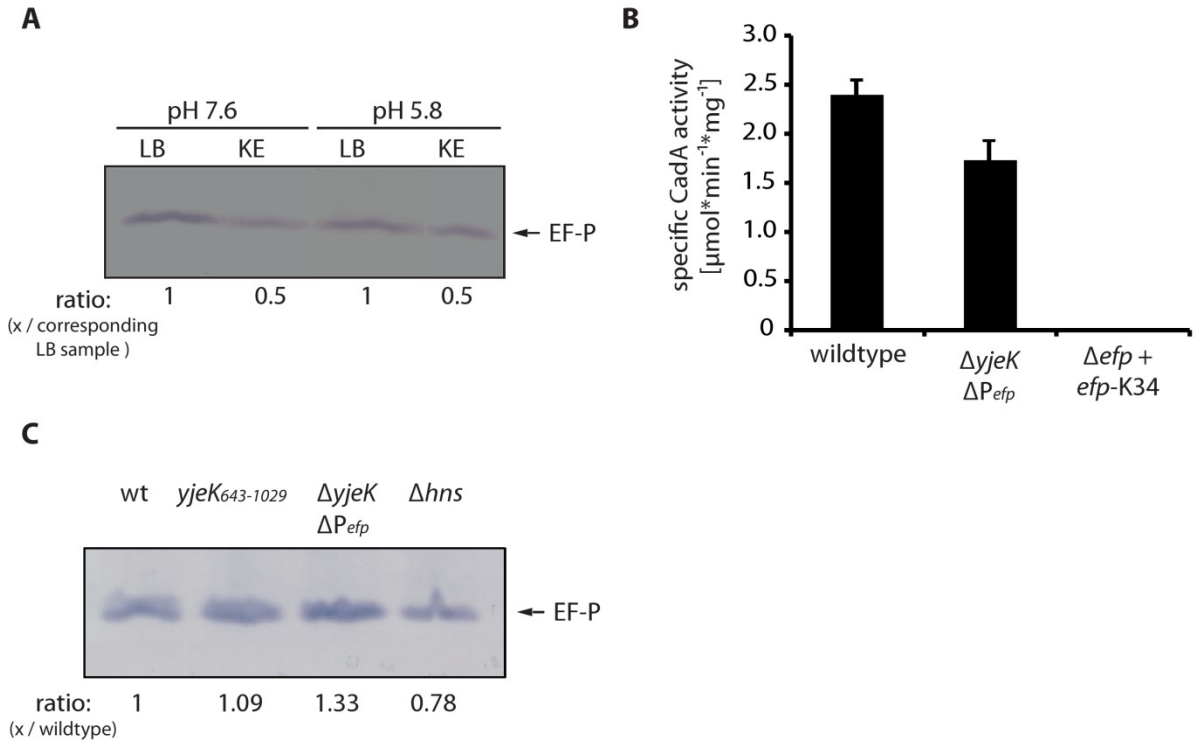


Fig. 29: EF-P amounts in LB and KE medium.

A) Western Blot analysis of cells grown in LB or KE medium using EF-P specific antibodies. Cells of BW25113 were first cultivated microaerobically in LB or KE medium under non-inducing conditions (pH 7.6) and were then shifted to inducing conditions (pH 5.8, with 10 mM lysine in KE) for 1 h. Quantifications were made by using the program ImageJ.

B) CadA phenotype of BW25113 wildtype, JW4106 (Δ*yjeK,* including *efp* promoter) and JW4107 (Δ*efp*) harboring plasmid pQE70-ECO-*efp* K34A. Cells were grown in KE minimal medium under inducing conditions (pH 5.8, 10 mM lysine). Strain JW4106 harbors a start to stop deletion of *yjeK*, including the *efp* promoter.

C) Western Blot analysis with EF-P specific antibodies. Cells of strain BW25113 wildtype (wt), SU1 (Δ$yjeK_{643\text{-}1029}$), JW4106 (Δ*yjeK*, including *efp* promoter), and JW1225 (Δ*hns*) were grown in KE minimal medium under inducing condtions (pH 5.8, 10 mM lysine). 10 µl of cells adjusted to OD_{600} = 10 were loaded per lane. Quantifications were made by using the program ImageJ.

3.11 Role of Hfq in the Cad system

The Gad system (AR2) employs not only regulatory proteins like GadX and GadW, but also the small regulatory RNAs GadY and GcvB, participating in the very complex regulation of the stress system (Jin *et al.*, 2009; Opdyke *et al.*, 2004). The protein Hfq is known to interact with and to stabilize small RNAs. To examine, if small RNAs are occupied in the Cad system as well, an *hfq* mutant was used, which was described before (Muffler *et al.*, 1996). The Δ*hfq* mutant, wildtype MG1655 and the mutant harboring plasmid pBAD24-Hfq-His_6 were cultivated in KE medium (pH 5.8 with 10 mM lysine), harvested at their exponential growth phase and the CadA activities were determined. Interestingly, the *hfq* mutation led to a highly reduced CadA activity, which could be restored by complementation (Fig. 30A). When the strains were cultivated in LB medium, the Δ*hfq* mutant regained 60% of the wildtype CadA activity (Fig. 30A).

In a parallel approach, $hfq_{1\text{-}230}$ was deleted in strain MG-CR (MG1655 Δ*lacZ* Δ*cadBA* P_{cadBA}-*lacZ*), too. Since the start codon of *hflx,* encoding for a GTPase, is located only 75 bp downstream of the stop codon of *hfq*, only nucleotides 1-230 were exchanged by chloramphenicol. Thus, secondary effects could be excluded. Additionally, $hflx_{1\text{-}750}$ was exchanged by a kanamycin resistance cassette in strain MG-CR. Afterwards, cells of strains MG-CR, MG-CL-12-*hfq* and MG-CL-12-*hflx* were cultivated under inducing conditions (KE medium, pH 5.8 with 10 mM lysine), and β-galactosidase activities were measured. Again, the *hfq* mutation had a strong effect on *cadBA* expression (Fig. 30B). However, Δ*hflx* cells revealed wildtype-like β-galactosidase activities, excluding Hflx to play a role in the Cad system.

Next, it was explored if Hfq is crucial for *cadC* expression. To this end, MG1655 wildtype cells and Δ*hfq* cells were cultivated under inducing conditions in KE minimal medium. After preparation of the membrane vesicles of these cells, 150 µg of the total protein amount were loaded onto a SDS gel and a Western Blot was performed using specific CadC antibodies. Indeed, no CadC could be detected in the mutant (Fig. 30C).

Guisbert *et al.* previously showed by microarray analysis that Hfq plays a role for EF-P expression (Guisbert *et al.*, 2007). To verify that the EF-P amount is altered in a Δ*hfq* background, 40 ml of cells of wildtype MG1655 and the Δ*hfq* mutant were incubated in inducing KE medium (pH 5.8 with 10 mM lysine). After harvesting, 10 µl of cells (OD_{600} = 10) of each sample were loaded onto a SDS gel, and a Western Blot was performed using specific antibodies against EF-P. However, no difference could be observed in the EF-P

amounts of the wildtype and the Δ*hfq* mutant (Fig. 30D), excluding any direct effect of Hfq on *efp* expression.

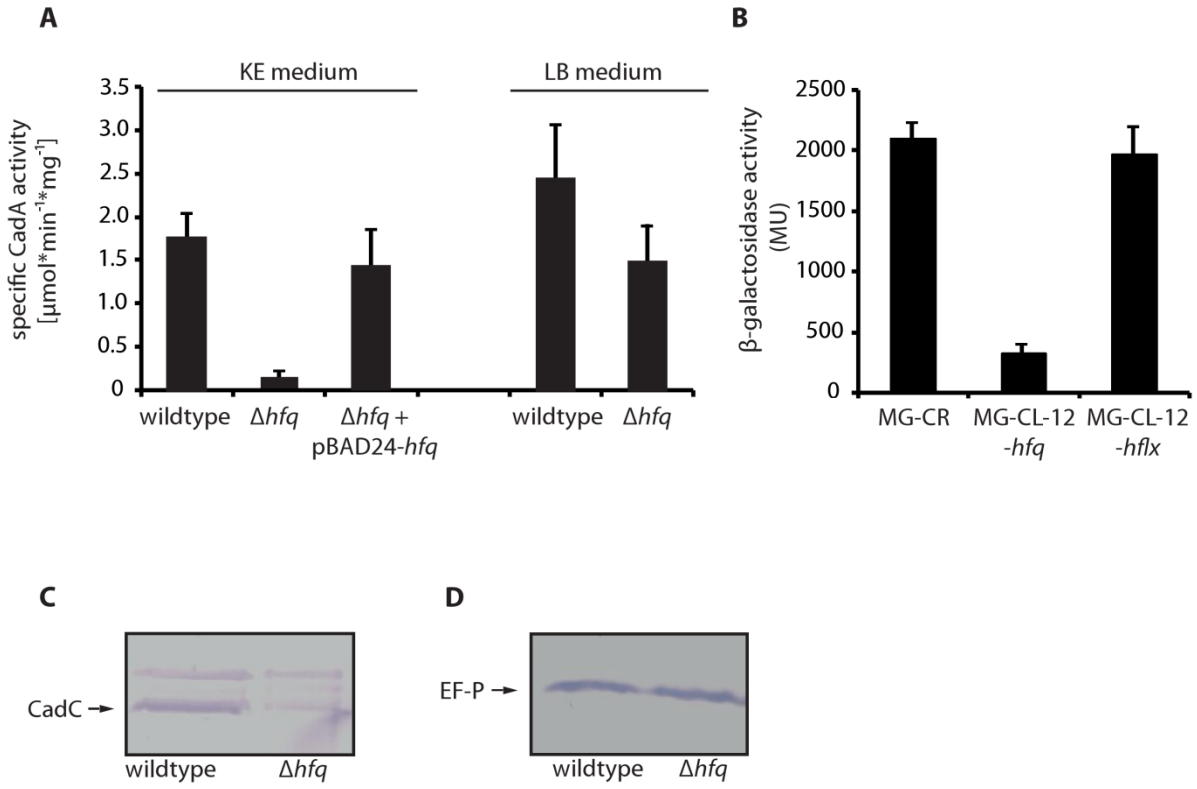


Figure 30: Hfq is involved in *cadBA* expression.

A) CadA phenotype of the Δ*hfq* mutant in KE minimal and LB medium. Cells were grown under inducing condtions (pH 5.8 and 10 mM lysine in KE medium).

B) *cadBA* expression in the Δ*hfq* mutant and Δ*hflx* mutant. Cells were grown in KE minimal medium under inducing conditions (pH 5.8, 10 mM lysine).

C) Western Blot analysis of CadC in the wildtype and the Δ*hfq* mutant using CadC specific antibodies.

D) Western Blot analysis of EF-P in the wildtype and the Δ*hfq* mutant using EF-P specific antibodies.

As mentioned above, the Δ*hfq* mutant regained 60% of the wildtype CadA activity, when cultivated in LB medium (Fig. 30A). This raises the question, if Hfq is needed for expression of the EF-P modifiers YjeA and YjeK. *yjeA* and *yjeK* mutants showed a similar *cadBA* expression pattern in KE medium and in LB medium comparable to the *hfq* mutant: No *cadBA* expression could be observed in KE medium under non-inducing conditions, but 80%

(Δ*yjeA*), 30% (Δ*yjeK*) or 60% (Δ*hfq*) expression of the wildtype in LB medium under non-inducing conditions (Fig. 28A, 30A). Therefore, cells of MG1655 and the Δ*hfq* mutant were transformed with plasmids pBAD24-*yjeA* and pBAD24-*yjeK,* respectively.

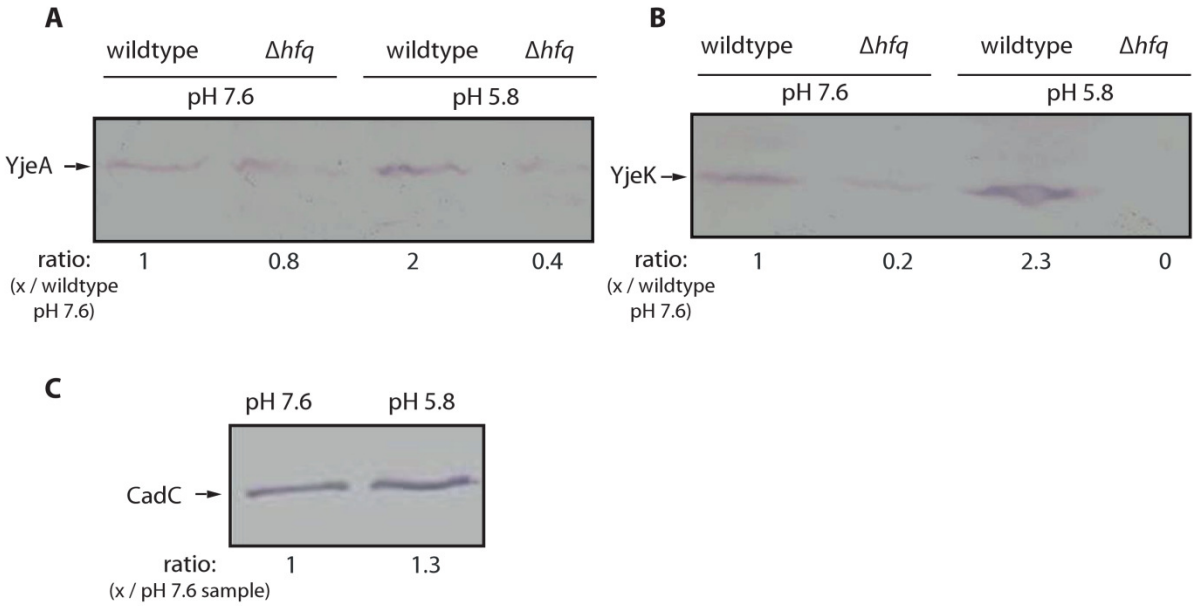


Figure 31: Regulation of modified EF-P.

A) Effect of *hfq* mutation on *yjeA* and **B)** *yjeK* expression. Cells were grown in KE medium under non- (pH 7.6) and inducing conditions (pH 5.8, 10 mM lysine). Western Blot analysis were prformed using Penta-His anti-Mouse antibodies against His_6-YjeA and His_6-YjeK. 10 µl of cells adjusted to OD_{600} = 10 were loaded per lane. Quantifications were made by using the program ImageJ.

C) Western Blot analysis of CadC using a Penta-His anti-Mouse antibody. Cells of MG1655 Δ*cadC* harboring pET16b-CadC were cultivated in LB under non-inducing conditions (pH 7.6) and were then shifted to inducing conditions (pH 5.8, 10 mM lysine) for 1 h. 150 µg of membrane vesicles were loaded per lane. Quantifications were made by using the program ImageJ.

Cells were first cultivated in KE medium under non-inducing conditions (pH 7.6) and then, at an OD_{600} of ≈ 0.5, shifted to inducing conditions (KE medium, pH 5.8 with 10 mM lysine, no shaking) for 1 h. 10 µl of cells adjusted to an OD_{600} of 10 were loaded onto a SDS gel. The Western blot was performed by using specific antibodies against the His-tag. Strikingly, both YjeA and YjeK protein levels were highly reduced in the Δ*hfq* mutant (Fig. 31A, B), indicating an effect on *yjeA* and *yjeK* expression by Hfq. Additionally, YjeA and YjeK levels were nearly twice as much for cells grown for 1 h under inducing conditions as for cells

grown only under non-inducing conditions (Fig. 31A, B). To further investigate, if consistently not only *cadC* transcription, but also translation is enhanced under stress conditions leading to the CadC "boost" at pH 5.8, strain MG1655 Δ*cadC* was transformed with plasmid pET16b-CadC. As *cadC* was set under the control of the T7 promoter in pET16b, transcriptional regulation could be excluded. Cells were cultivated aerobically in KE or LB medium, pH 7.6, and were subsequently shifted for 1 h to inducing conditions. After preparation of the membrane vesicles, 100 µg of the total protein amount were loaded onto a SDS gel, and a Western blot analysis was employed using specific CadC antibodies. Indeed, the CadC amount was slightly enhanced, so that cells exposed to acidic stress in KE as well as in LB medium showed 1.3 times higher CadC levels (Fig. 31C, for KE data no shown).

Given that higher amounts of YjeK and YjeA lead to enhanced EF-P modification and thus to enhanced synthesis of EF-P dependent proteins, these results could provide the first hint that the modification of EF-P is stress dependently regulated.

4. Discussion

4.1. EF-P and its role in translation

EF-P is highly conserved in all kingdoms of life and present in all bacteria. Despite its universal conservation, the biological function of EF-P has remained enigmatic so far. It has been highly discussed for years, if EF-P and its eukaryotic orthologue eIF5A act as initiation or elongation factors. In this study, the ability of EF-P to influence first peptide bond synthesis was further characterized in more detail.

In contrast to the in vitro data, it turned out that an exchange of amino acid two (following the initiator methionine) in LacZ did not lead to an EF-P dependent increase or reduction of protein synthesis in vivo (section 3.4.1), excluding a general role of EF-P in translation initiation. However, protein synthesis varied greatly in the *efp* mutant and as well in the wildtype dependent on the second amino acid, showing high expression rates with phenylalanine and low expression rates with glycine at amino acid position two. The ribosomal petidyltransferase does not condense all aminoacyl-tRNAs equally (Rychlik *et al.*, 1970; Symons *et al.*, 1978), and it has been reported before that not only the amino acid itself, but also its codon is critical for protein expression rates (Bivona *et al.*, 2010; Looman *et al.*, 1987). Thus, translation initiation is a crucial rate limiting step in translation (Preiss & Hentze, 2003). This effect was also observed, when *cadC* was cloned into vector pBBR-MCS5 with variable codons at position two: When *cadC* contained the codon for a glycine after the start codon, the sequence AUG-GGU resulted in about 10 times higher CadC amounts than AUG-GGG (Marie-Charlotte Lenoir, unpublished). This so-called codon bias is based on the fact that some codons are more often used than others (Konigsberg & Godson, 1983; Robinson *et al.*, 1984; Sharp & Li, 1987). Codon bias leads to faster and higher expression rates for mRNAs containing major codons (like GGU), and to reduced expression of mRNAs containing minor codons (like GGG) (Brockmann *et al.*, 2007; Sorensen *et al.*, 1989). Given that a high protein amount is often desired, for example for protein purification or increased detection of fluorescence signals (Bivona *et al.*, 2010; Haas *et al.*, 1996), it is very useful to know that protein amounts can be optimized with regard to the second codon of the gene sequence. However, it can be excluded that EF-P acts as ribosomal helper protein for

less frequent codons, as the ribosomal stalling in *efp* mutants happens codon independently at proline stretches (Fig. 20A).

Previous studies supported a role for eIF5A in translation elongation, as ribosome profiles of eIF5A-depleted yeast cells did not show reduced polysome sizes or an increase in the 80S peak, which would be characteristic for an impaired translation initiation (Kang & Hershey, 1994; Saini *et al.*, 2009). In addition, modified eIF5A was identified to interact with elongation factors eEF1A and eEF2 and with the 80S ribosome (Jao & Chen, 2006; Zanelli *et al.*, 2006). However, this study revealed that EF-P is not important for translation of *lacZ*, *cadA*, *cadB* or *poxB*, thereby ruling out a general role of EF-P in translation. Accordingly, former reports suggested that EF-P and eIF5A are only needed for a small subset of mRNAs (Kang & Hershey, 1994; Navarre *et al.*, 2010; Yanagisawa *et al.*, 2010). It has been also considered that EF-P and eIF5A work in a field different to translation, like mRNA transport and mRNA decay (Bevec *et al.*, 1996; Hofmann *et al.*, 2001; Zuk & Jacobson, 1998). Although additional functions for EF-P or eIF5A cannot be excluded, the crystal structure of EF-P bound to the ribosome (Blaha *et al.*, 2009), the L-shaped structure of EF-P resembling the characteristic design of tRNAs (Hanawa-Suetsugu *et al.*, 2004), and the results obtained in this study claim a dominant role for EF-P in translating a small subset of proline-rich proteins.

4.2. Ribosomal stalling at a cluster of three prolines and additional stalling sequences

In CadC, a cluster of three consecutive prolines can be found in a long linker region between the DNA-binding domain and the transmembrane domain (Fig. 17). Proline is unique in that it is not an amino acid, but an imino acid (N-alkylamino acid), leading to very slow dipeptide formation accommodating the ribosomal A-site (Pavlov *et al.*, 2009). The secondary α-amino group of proline is an inefficient acceptor during peptide-bond formation (Fig. 32) (Muto & Ito, 2008; Pavlov *et al.*, 2009). Thus, translation of proteins containing three consecutive prolines resulted in ribosome stalling in vivo and in vitro in the absence of EF-P (Fig. 17, Fig. 21). The stalled ribosome is recognized by EF-P, which probably binds in a manner such that the lysinylated-lysine 34 extends into the peptidyltransferase center (PTC) of the ribosome and stimulates peptide-bond formation between the prolyl-$tRNA^{Pro}$ substrates (Fig. 32).

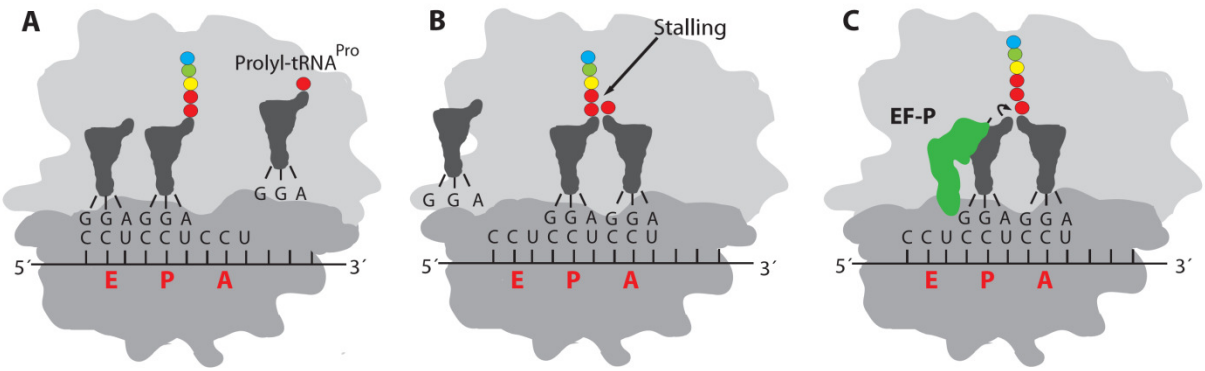


Fig. 32: EF-P alleviates ribosomal stalling at poly-proline stretches.

A, B) Binding of a third consecutive Pro-tRNA to a translating ribosome results in translational stalling.

C) The 3xPro-stalled ribosome is recognized by EF-P, which binds to the ribosome and stimulates peptide-bond formation between the A- and P-site proline substrates (arrowed). Thus, the elongation arrest is alleviated and translation can continue.

Interestingly, the crystal structure of EF-P bound to the 70S ribosome revealed that EF-P is located between the ribosomal P- and E-site (Blaha *et al.*, 2009), standing in good accordance with the proposed model above (Fig. 32C). Furthermore, EF-P interacts with the ribosomal L1 protein, which builds the highly mobile L1 stalk of the 50S subunit with three 23S rRNA helices (Blaha *et al.*, 2009). The L1/EF-P interaction leads to the transfer of the stalk into the E-site (Blaha *et al.*, 2009), thereby playing a role for the translocation of tRNAs from the P- to the E-site and the release of empty tRNAs from the E-site (Trabuco *et al.*, 2010). Another reason for stalling alleviation could be a conformational change of the ribosomal PTC or the right positioning of the Pro-Pro-Pro peptide chain triggered by EF-P.

For ribosomal stalling, the nascent peptide chain also seems to be important. Some nascent peptides, like SecM or TnaC, regulate their own translation rate via a special stalling motif (Tanner *et al.*, 2009). As the nascent peptide chain has to transit the aqueous exit tunnel of the 50S subunit (Nissen *et al.*, 2000), before it is released to the cytoplasm, various events can occur to trigger ribosomal stalling. For example, residues of the ribosomal tunnel and the nascent chain can interact, leading to conformational changes in the nascent chain and/or in the ribosome (Woolhead *et al.*, 2006). In addition, the conformation of the peptidyl t-RNA can be altered resulting in abolished translocation (Woolhead *et al.*, 2006). In the case of SecM, the ribosome stalls at a 17 amino acid long sequence containing residues which are

crucial for the stalling process: FXXXXWIXXXXGIRAGP^{166} (Nakatogawa & Ito, 2002). The stalling can be released, when the signal peptide of SecM is "pulled" out of the ribosomal tunnel by components of the Sec apparatus (Butkus *et al.*, 2003). By contrast, translational stalling is not abolished under conditions with insufficient secretion. The stalling then results in changes of the secondary structure of the mRNA and in expression of the downstream gene *secA* (Nakatogawa & Ito, 2001), which encodes for a preprotein translocase subunit (Cabelli *et al.*, 1988). Similarly to the SecM stalling motif, also TnaC, the leader peptide of the tryptophanase operon (Konan & Yanofsky, 1997), contains a C-terminal sequence crucial for stalling: WXXXDXXXXXXXP^{stop} (Gong & Yanofsky, 2002). In the absence of tryptophan, the ribosome stalls leading to an antiterminator stem loop on the mRNA and to expression of the downstream genes including tryptophan producing enzymes. In the presence of tryptophan however, the stalling is abolished and a terminator stem loop is built. As a consequence, downstream genes are not expressed (Buchan & Stansfield, 2007; Yanofsky & Horn, 1994). The stalling motifs of SecM and TnaC both contain a C-terminal proline, which is very crucial for the translational arrest (Tanner *et al.*, 2009). Furthermore, an Asp, Glu or Pro upstream of the Pro-Stop of protein YbeL leads to enhanced SsrA-tagging (Hayes *et al.*, 2002). SsrA is also known as transfer-messenger RNA (tmRNA) and it can recognize and relief stalled ribosomes by a special mechanism, in which the stalled aminoacyl-tRNA at the A-site is replaced by an Ala from the aminoacylated tmRNA. The tmRNA then acts as template for a short peptide tag. The hybrid protein is released from the ribosome and afterwards degraded by cellular proteases (Keiler *et al.*, 1996; Tu *et al.*, 1995). This tagging method was also used by Tanner *et al.* to identify additional stalling peptides (Tanner *et al.*, 2009). Interestingly, isolated peptides contained the consecutive amino acids PPP, PPG and APP, respectively. The same amino acid clusters were identified to cause stalling in *cadC* (PPP, PPG) and *flhC* (APP) in the absence of EF-P in this study (Fig. 27A, C). Consistent with the stalling caused by GPP in *cadC* (Fig. 27C), Muto *et al.* could show that a glycine tRNA^{Gly} occupying the ribosomal P-site and a prolyl-tRNA^{Pro} occupying the ribosomal A-site disrupt the peptidyltransferase reaction (Muto & Ito, 2008). Accordingly, Doerfel *et al.* confirmed an EF-P mediated release of ribosomal stalling at PPG sequences (Doerfel *et al.*, 2013). Taken together, it is obvious that EF-P recognizes specific clusters of three amino acids, mostly harboring at least two prolines. The strongest stalling effect was observed with three consecutive prolines in this study. For future studies it will be important to examine all possible EF-P related stalling sequences in more detail.

Besides the consecutive prolines, other residues of the nascent chain were identified to interact with ribosomal residues leading to a conformational change of the peptide in the exit tunnel, and – together with the proline at the A-site – to ribosomal stalling (Woolhead *et al.*, 2006). The essential amino acids of the nascent peptide chain possess preferably large side chains, as tryptophan or phenylalanine, and lay 11 to 15 amino acids upstream of the stalling point (Woolhead *et al.*, 2006). Interestingly, *E. coli* CadC contains a tryptophan 14 amino acids upstream of Pro120. By contrast, *Vibrio* CadC lacks not only the prolines, but also the tryptophan (Fig. 22A).

All these investigations stand in good accordance with section 3.9 of this study, in which stalling parameter in addition to the prolines were examined in more detail. It was shown that the effect of an *efp* mutation is more severe, when the prolines are located in a long unstructured region (Fig. 26). Comprising the facts described above, it can be speculated, why a helix upstream of the stalling region could reduce ribosome stalling. One possible reason is that the long unstructured region, being flexible, simplifies interaction with the ribosomal tunnel, leads to conformational changes of the nascent chain in the tunnel and/or the ribosome and thus increases ribosomal stalling. Accordingly, a shorter unstructured region followed by a helix or a β-strand - as it is the case in HisD, which is EF-P independent – could lead to more rigidity of the peptide and thus to less interaction with the ribosome resulting in reduced ribosomal stalling.

As mentioned above, the accommodation step (binding of the aminoayl t-RNA to the A-site) and the peptidyl transfer are very slow, when a proline is incorporated in the nascent polypeptide chain (Pavlov *et al.*, 2009). Consequently, three consecutive prolines lead to even higher ribosomal stalling, which requires the help of an auxiliary protein like EF-P to continue translation. The interaction of EF-P with ribosomal components like the L1 stalk could then result in conformational changes of the ribosome, the nascent peptide chain or the peptidyl-tRNAs, which is important to continue translation elongation. Without EF-P, only a short peptide is built, before the ribosomal complex is disassembled (see Fig. 21 and 25). Thus, the protein concentration drops to a minimum, as it was experienced in the case of CadC. Additionally, not only the amino acid itself, but also codon bias plays a significant role for ribosomal stalling (Hayes *et al.*, 2002; Konigsberg & Godson, 1983). The proline codon CCC is rarely used in the *E. coli* genome, thus also its corresponding t-RNA is diminished in the cell leading to even slower proline incorporation as obtained with the other three codons (Pavlov *et al.*, 2009). Interestingly, *cadC* contains three consecutive prolines with the codon CCU, which is the second rarest codon for proline in the *E. coli* genome, whereas the EF-P

independent *hisD* contains three CCG prolines – a codon, which can be found quite frequently in the genome (Sharp & Li, 1987). However, also the EF-P dependent NlpD protein contains three prolines with the CCG codon. Therefore, it is very likely that the codon bias contributes to ribosomal stalling with some RNAs, but not with others depending on additional stalling factors.

In conclusion, three consecutive prolines can induce ribosomal stalling, which is alleviated by EF-P. In addition, other stalling sequences can be identified, like APP, PPG and GPP, but three consecutive prolines have the strongest effect. Additionally, also the proline preceeding amino acids can be essential for ribosomal stalling, as the ribosomal tunnel can interact with residues of the nascent polypeptides. Furthermore, the codon bias can contribute to the rate determing step of accommodation of the aminoacyl t-RNA to the A-site. Accordingly, different stalling motifs can lead to distinct stalling events and thus to different translation rates of proteins (Ito *et al.*, 2010).

4.3. The CadC/LysP balance model

It has been discussed previously that EF-P as well as eIF5A help to synthesize only certain proteins (Bartig *et al.*, 1992; Roy *et al.*, 2011). This raises the question, why *cadC* is one of the specific targets of EF-P. Further studies revealed that the amount of CadC is important for regulated expression of the *cadBA* operon (Küper & Jung, 2005). Having demonstrated that modified EF-P is crucial for CadC translation, the importance of this new control mechanism for the regulation of the Cad-module was investigated in more detail. The EF-P independent CadC-PPPIP/AAAIS variant resulted in about three times more CadC molecules as wildtype CadC leading to partial *cadBA* expression even in the absence of stimulation (Fig. 23A, B). Accordingly, expression of plasmid-encoded CadC at different levels corroborated the strong correlation between the increased CadC copy number and a stimulus-independent response (Fig. 24A). As depicted in Fig. 2, activation of CadC is dependent on its co-sensor LysP. Overproduction of LysP prevents *cadBA* expression under all conditions (Neely & Olson, 1996) indicating that the copy number of LysP is also crucial for a stimulus-dependent response.

Together with the new data on CadC levels, a balance model can be proposed for the Cad-system (Fig. 33): In wildtype cells the CadC/LysP ratio is balanced (Fig. 33A, middle panel). In response to the two stimuli, low pH and lysine, the balance is shifted in favor of activated

CadC leading to *cadBA* expression (Fig. 33A, left panel). Under non-inducing conditions, expression of *cadC* is lower than under inducing conditions (Krin *et al.*, 2010).

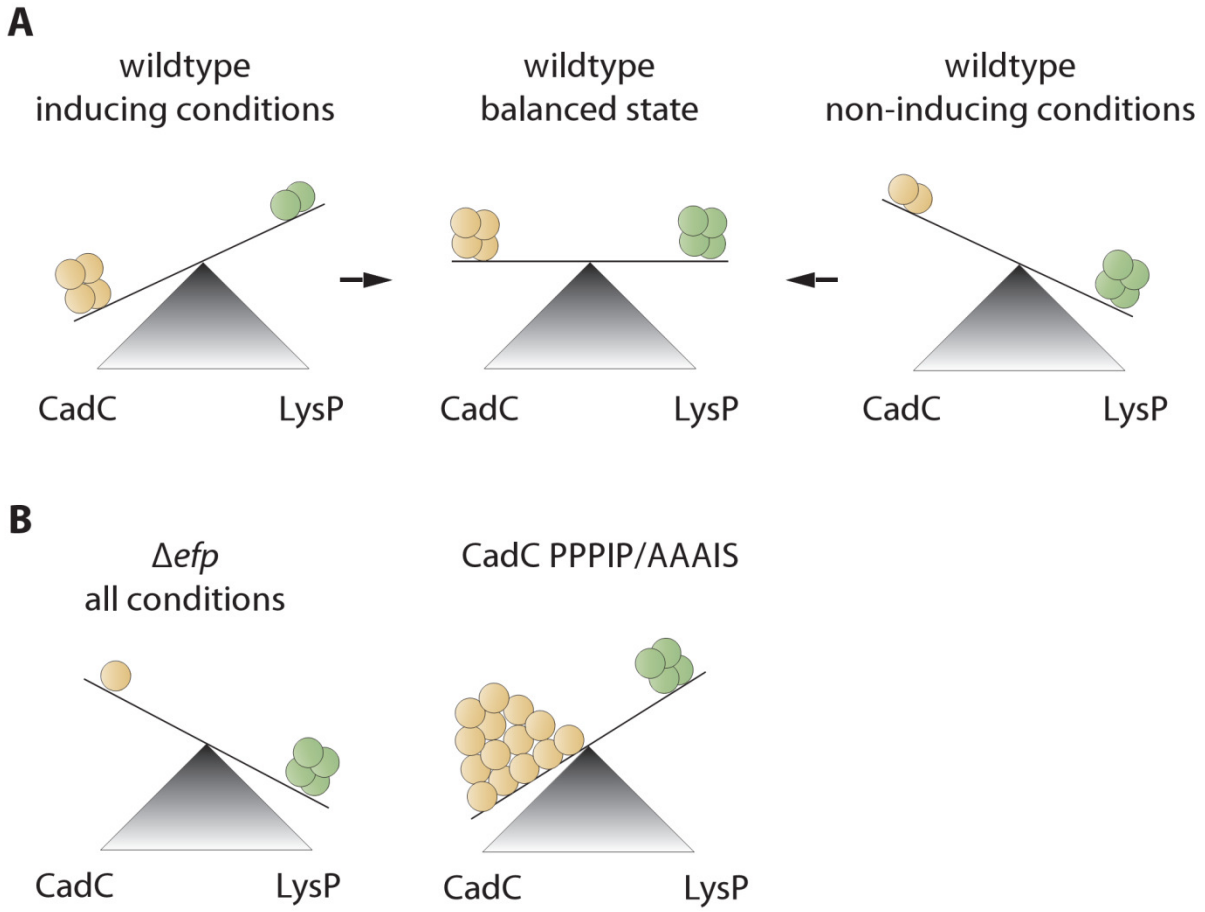


Fig. 33: The CadC/LysP balance model.

A) In the wildtype, the balance can shift in favor of CadC under inducing conditions (left panel) and in favor of LysP under non-inducing conditions (neutral pH medium without lysine, right panel). The CadC/LysP ratio is balanced (middle panel).

B) An *efp* deletion leads to the shift in favor of LysP (left panel). The EF-P independent variant CadC PPPIP/AAAIS leads to the shift in favor of CadC (middle panel).

Simultaneously, *lysP* expression is enhanced due to the lack of lysine repression (Ruiz *et al.*, 2011), thus leading to a shift of the balance in favor of LysP and to abolished *cadBA* expression (Fig. 33A, right panel). In cells lacking EF-P, the CadC/LysP ratio is imbalanced to disfavor CadC and therefore *cadBA* expression is significantly reduced (Fig. 33B, left panel). In cells harboring the EF-P independent variant CadC-PPPIP/AAAIS, the CadC/LysP ratio is also imbalanced but in favor of CadC, resulting in a partial stimulus-independent

cadBA expression (Fig. 33B, middle panel). Therefore, the translational control of CadC by active EF-P represents a novel mechanism to tightly fine-tune the protein copy number.
Interestingly, *Vibrio* lacks not only the CadC inhibitor LysP, but also the proline cluster (section 3.6.1). As these bacteria do not have to deal with the CadC/LysP ratio, they also do not need the proline cluster for EF-P mediated copy number control.

Taking the Northern Blot results of Neeley *et al.* into account (Neely & Olson, 1996), the CadC/LysP balance model can help to explain the varying stress response times of the *cad* operon obtained by three different start conditions:
Firstly, when *E. coli* cells (Δ*cadA*) were grown in neutral pH minimal medium with lysine, before they were shifted to inducing conditions (pH 5.8 with 10 mM lysine), *cadB* mRNA was detected 4 min after the shift (Neely & Olson, 1996). *lysP* expression is repressed by lysine (Neely & Olson, 1996; Ruiz *et al.*, 2011). Thus in lysine containing medium, the level of LysP is lower than in medium without lysine. Additionally, in neutral pH medium *cadC* is expressed, but to a lower extent than in low pH medium (Dell *et al.*, 1994; Krin *et al.*, 2010). Furthermore, lysine is transported into the cell by LysP resulting in reduced CadC inhibition (Tetsch *et al.*, 2008). When cells are shifted to acidic medium with lysine, expression of *cadC* is enhanced, but not the expression of *lysP*. CadC can form dimers as its levels are increased due to the low pH and thus transfer the stress signal to the *cadBA* operon (Eichinger *et al.*, 2011; Haneburger *et al.*, 2011; Tetsch *et al.*, 2011).
Secondly, the same response time was obtained, when cells were first grown in low pH medium without lysine (Neely & Olson, 1996). In low pH medium without lysine, the LysP level is increased in comparison to medium with lysine, because its expression lacks lysine repression. Furthermore, CadC levels are higher due to the low pH. Thus, both levels of LysP and CadC are increased in pH 5.8 medium, but the CadC/LysP ratio is comparable to that in the pH 7.6 medium containing lysine. Consequently, this leads to the same short response time of *cadB* expression.
Finally, when cells were first grown in neutral pH medium without lysine, it took 15 min until *cadB* expression could be detected (Neely & Olson, 1996). This raises the question, why two stimuli (lysine and low pH) lead to nearly four times slower stress response than one stimulus (lysine or low pH). In neutral medium without lysine, LysP levels are high, as no lysine repression occurs. By contrast, CadC levels are lower than under acidic conditions, thus all CadC molecules are inhibited by LysP, an excess of free LysP is still available and the balance is shifted in favor of LysP (Fig. 33A, right panel). Thus, the CadC/LysP ratio differs

from the ratio described above. Therefore, more CadC molecules must be built for sufficient *cadBA* expression after the shift to overcome higher LysP levels. Consequently, a two stimuli induction takes more time than induction by only one stimulus.

Another interesting aspect is the de novo synthesis suggested by Neely *et al.* (Neely & Olson, 1996). Following this line, EF-P could be important for the new synthesis of an active form of CadC under inducing conditions resulting in the expression of the *cadBA* operon. This leads to the question, whether EF-P itself is regulated under specific stress conditions. In general, regulation of gene expression is achieved by several different ways, including regulation of transcription via various transcription activators, repressors and sigma factors (Browning & Busby, 2004; Mata *et al.*, 2005). The concentration of proteins depends also on mRNA stability, the translation rate and protein degradation rate (Mata *et al.*, 2005). Amongst others, translation is affected by ribosomal stalling, secondary mRNA structures, the codon bias and the number of available ribosomes, tRNAs and translation factors (Brockmann *et al.*, 2007; Konigsberg & Godson, 1983; Kozak, 2005; Mata *et al.*, 2005). One EF-P molecule per 10 ribosomes exists in the cell (An *et al.*, 1980), which stands in good accordance to the number of other translation factors (Cole *et al.*, 1987). However, the number of active EF-P could be much lower as the protein has to be posttranslational modified by the two enzymes YjeK and YjeA, which have rather low enzymatic activities (Behshad *et al.*, 2006; Brevet *et al.*, 1995). Therefore it is possible, that under specific stress conditions the modification of EF-P increases resulting in higher translation rates for the EF-P target mRNAs. Only few reports exist about translation factors, which are modified and activated under certain stress conditions (Blais *et al.*, 2004; Giuliodori *et al.*, 2007; Milon *et al.*, 2006; Spriggs *et al.*, 2010). This study revealed that the amounts of YjeA and YjeK are doubled, when cells were grown microaerobically in medium with low pH and lysine (Fig. 31A, B). In addition to that, also the translation of *cadC* was slightly increased under inducing conditions (Fig. 31C). Thus, a stress dependent modification of EF-P is very likely. However, whether this effect is due to the switch from neutral to acidic medium or from oxygenated medium to medium with low oxygen needs to be further investigated.

Furthermore, this study indicates that the amounts of CadC, YjeA and YjeK, but not of EF-P, are decreased in *hfq* mutants. Hfq acts as RNA binding chaperone, stabilizes small non-coding RNAs (sRNA) and facilates mRNA/sRNA interactions, thereby causing stabilization or degradation of mRNAs (Valentin-Hansen *et al.*, 2004; Zhang *et al.*, 2003). This result leads to speculations, if the mRNAs of *yjeA* and *yjeK* are controlled by small regulatory RNAs.

Interestingly, computational analysis predicted an yet unknown small non-coding RNA in the genomic region of *yjeA* (Rivas *et al.*, 2001). However, if this small RNA really influences the stability of the *yjeA* mRNA, or if additional proteins are involved causing the effects of the *hfq* mutant, needs still to be uncovered in future experiments.

4.4. EF-P and its importance in plant and mammalian diseases

The deletion of *efp* or its orthologues leads to several different phenotypes. Having demonstrated that EF-P alleviates translational stalling at polyproline-stretches in *E. coli,* it is now possible to address some of the proteins, which could be responsible for the resulting effects in *efp*, *yjeK* and *yjeA* mutants.

Peng *et al.*. determined the phenotype of a deletion mutant (Δ*chvH*) in *Agrobacterium tumefaciens* (Peng *et al.*, 2001). *chvH* encodes for a homologue to *E. coli efp* and clusters with *poxA* (*yjeA*) and *kamA* (homologue to *yjeK*) in the genome. The *chvH* deletion leads to avirulence and other pleiotropic effects, which can be complemented by *E. coli* EF-P (Peng *et al.*, 2001). *A. tumefaciens* is very particular as it is able to transfer bacterial DNA into the plant host, followed by integration of the DNA into the plant genome and formation of crown galls (Bevan & Chilton, 1982). The tumor-inducing plasmid Ti harbors the VirA/VirG two-component system, which is responsible for the expression of additional *vir* genes (Leroux *et al.*, 1987; Ronson *et al.*, 1987; Winans *et al.*, 1986). Comparable to CadC, VirG and VirA are only expressed to significant levels, when cells respond to environmental stress conditions like low pH (Peng *et al.*, 2001; Winans *et al.*, 1988). Interestingly, protein amounts of VirA, VirG and other tested Vir proteins are reduced in the *chvH* mutant (Peng *et al.*, 2001). VirA contains the amino acid sequence PPG, which was previously shown to cause ribosomal stalling (Tanner *et al*, 2009; Doerfel et al, 2013; this study). Therefore it can be speculated, whether the reduced protein level of the hisidine kinase VirA leads to the observed impaired production of the Vir proteins in the *chvH* mutant. In addition, also VirB9 (PPQP), VirB10 (PPS, PPA, PPP, PPT), VirB11 (PPT, PPQ), VirE2 (PPP, PPI) and VirD2 (PPD, PSPP) contain a cluster of at least two prolines, indicating that also these amino acid sequences could contribute to ribosomal stalling and to decreased protein levels in the *chvH* mutant. Furthermore, the deletion of *chvH* reveals an increased production of a so far uncharacterized protein (Peng *et al.*, 2001). As the transcriptional regulator VirB, which is phosphorylated by VirA, shows a highly reduced protein amount in the *chvH* mutant (Peng *et al*, 2001), the unknown protein perhaps acts as repressor of VirB. Taken together, it seems very likely that

ChvH fine-tunes the levels of *vir* regulon components for adequate stress response, which is comparable to the Cad system.

In the U.S., approximately 9.4 million people contract foodborne diseases each year, resulting in over 1,300 deaths, of which 11% derive from *Salmonella enterica* (Mead *et al.*, 1999; Scallan *et al.*, 2011). These bacteria are able to overcome the host immune system and to multiply in varieties of mammalian cells while residing in so-called *Salmonella*-containing vacuoles (SCV) (Bakowski *et al.*, 2008; Prost *et al.*, 2007). Via horizontal gene transfer, *S. enterica* acquired several pathogenicity islands, which are essential for host invasion and survival in the new intracellular environment (Haraga *et al.*, 2008). Crucial for *Salmonella* virulence are two type III secretion systems, which are encoded on the pathogenicity islands 1 and 2 (SP-1 and SP-2) (Hansen-Wester & Hensel, 2001). Consistent with the *chvH* deletion in *A. tumefaciens*, deletions of *yjeK* or *yjeA* lead to attenuation in virulence and altered gene expression in *S. enterica*, including genes found on SPI-1 (Navarre *et al.*, 2010). The discovery of the role of EF-P in translation now opens up the possibility to determine proteins, which protein levels are very likely affected in these mutants. Several important virulence proteins contain polyproline-stretches, such as the type III secretion ATPase SsaN and the secretion system enhancer protein SseF, both of which are located on SPI-2. Moreover, EnvZ and PhoR, which regulate the transcription of SPI-1 and SPI-2 (Ellermeier & Slauch, 2008; Garmendia *et al.*, 2003), also contain polyproline-stretches. Furthermore, Zou *et al.* found out that the overexpression of the poorly characterized porin KdgM is the reason for the susceptibility to antibiotics, hypoosmotic stress and several detergents in *efp* mutants observed by Navarre *et al.* (Navarre *et al.*, 2010; Zou *et al.*, 2012). In the plant pathogen *Erwinia chrysanthemi*, KdgM is important for the transport of long oligogalacturonides derived from pectines (Blot *et al.*, 2002). Interestingly, *kdgM* expression is repressed by OmpR, a transcriptional activator that needs to be phosphorylated by the histidine kinase EnvZ for activity (Cai & Inouye, 2002; Condemine & Ghazi, 2007; Egger & Inouye, 1997). As mentioned above, EnvZ contains three consecutive prolines making it a promising target for EF-P.

Genomic analyses reveal an abundance of polyproline-stretches not only in bacteria, but also in archaea and eukaryotes. For example, ~1,000 (13%) yeast proteins contain at least three consecutive proline residues, whereas over 6,000 (20%) polyproline-containing proteins are present in humans. eIF5A has been shown to be involved in several human diseases. For example, Ruhl *et al.* described an interaction between eIF5A and the viral protein Rev, which is needed for HIV-1 mRNA transport out of the nucleus into the cytoplasm (Ruhl *et al.*,

1993). Thus it was discussed, if eIF5A also plays a role in mRNA transport. However, Rev contains a cluster of three consecutive prolines, thereby leading to the suggestion that eIF5A is rather important for the proper translation of Rev than for mRNA transport.

Furthermore, high levels of eIF5A could be detected in cancer cells making it an appropriate target as tumor marker (Balabanov *et al.*, 2007; Lee *et al.*, 2010; Preukschas *et al.*, 2012). High amounts of mutated p53 can be found in malign cells as well. Normally, the transcription regulator p53 leads to cell cycle arrest under genotoxic stress (Pellegata *et al.*, 1996). During this arrest, the cells have time to repair the DNA or to provoke apoptosis. Unregulated overproduction of p53 however leads to reduced cell cycle arrest, thus cells are able to divide themselves uncontrolled leading to the formation of tumors. Interestingly, p53 is a very proline rich protein containing a cluster of three consecutive prolines, but also several clusters of two prolines including the sequence PPG. eIF5A seems to mediate the translation of p53, thus contributing to a high level of mutated p53 in tumor cells.

Taken together, it could be demonstrated that EF-P and eIF5A play a major role for plant and mammalian diseases. Blocking of eIF5A/EF-P or of hypusination/lysinylation via pharmacological substrates will be of great importance for further studies in respect to find new antibiotics and therapies against bacterial infections, HIV and cancer (Balabanov *et al.*, 2007; Doerfel *et al.*, 2013; Hauber *et al.*, 2005; Lee *et al.*, 2010).

5. Summary

Enterobacteria have evolved several strategies to survive the acidic environment of the gastrointestinal tract. One of the acid stress resistance systems is the Cad system in *Escherichia coli*, which is induced by low pH and in the presence of external lysine. It consists of CadA, which catalyzes the decarboxylation of lysine to cadaverine, the lysine/cadaverine antiporter CadB and the pH sensing transcriptional regulator CadC. Moreover, the lysine permease LysP inhibits the induction of *cadBA* expression when lysine is absent, and the small histon-like molecule H-NS acts as repressor for both *cadBA* and *cadC* transcription. Additionally, a random mutagenesis approach revealed that a deletion in *yjeK* leads to highly reduced cadaverine production. YjeK acts as 2,3-lysine aminomutase (LAM) while catalyzing the isomerization of (S)-α-lysine to (R)-β-lysine. The truncated lysyl-tRNA synthetase YjeA uses (R)-β-lysine as substrate to post-translationally modify and to activate the translation elongation factor EF-P at a conserved lysine residue (K34). EF-P and its ortholog eukaryotic initiation factor 5A (eIF5A) have been investigated for more than thirty years, but their roles in translation remained enigmatic.

In this work the role of active EF-P in the Cad system was investigated in more detail. Reduced *cadBA* expression in Δ*yjeA*, Δ*yjeK*$_{642\text{-}1029}$ and Δ*efp* mutants was linked to impaired CadC translation. As the translation of *cadA* and *cadB* was EF-P independent, a general role of EF-P in translation could be excluded.

The identification of CadC as first direct target for EF-P in *E. coli* allowed further investigations on the role of EF-P in translation. Determining the β-galactosidase activities of CadC´-LacZ translational fusions of increasing CadC length in *efp*$^-$ and *efp*$^+$ cells revealed that EF-P is required for translation of the sequence found between codon 108 and 158 in *cadC*. This region comprises a cluster of three consecutive prolines (Pro120-Pro121-Pro122). Substitution of these prolines by alanines diminished EF-P dependency. Remarkably, cells harboring the CadC-PPPIP/AAAIS variant revealed *cadBA* expression even under non-inducing conditions. Thus, EF-P tightly controls the CadC copy number, which is crucial for stress dependent regulation of the Cad system.

In order to investigate the work mechanism of EF-P in more detail, EF-P independent CadC´-LacZ hybrids were employed to artificially introduce prolines. Three consecutive prolines were sufficient for EF-P dependency, regardless of the codon or the context. The proline-rich proteins AmiB, FlhC, Flk, NlpD, RzoR, TonB and UvrB also showed EF-P dependent expression. Thus, the recognition of three consecutive prolines by EF-P is a general mechanism and not limited to CadC. Dr. Agata Starosta of the group of Dr. Daniel Wilson (Gene Center, LMU Munich) confirmed ribosomal stalling at polyproline-stretches in samples lacking EF-P with in vitro translation assays.

Finally it was investigated, if EF-P expression and modification could be stress-dependently regulated. In this work first hints are given that the *efp* promoter contains a repressor site, and that expression of *yjeA* and *yjeK* is dependent on the pH of the medium and the presence of the small RNA binding protein Hfq. This leads to the suggestion that small regulatory RNAs are also involved in regulation of the EF-P modification enzymes.

In conclusion, the results obtained in this work reveal a new regulatory mechanism by EF-P dependent translation. 100-1000´s of polyproline rich proteins exist in bacteria, archaea and eukaryotes. Therefore, EF-P and its orthologs aIF5A and eIF5A most likely play an important role in the adjustment of copy numbers of proteins with different functions in all kingdoms of life.

6. Zusammenfassung

Enterobakterien haben eine Reihe von Strategien entwickelt, um saure Bedingungen im Gastrointestinaltrakt überleben zu können. Ein Resistenzsystem gegen Säurestress ist das Cad System von *Escherichia coli*, das durch niedrigen pH und extern vorhandenes Lysin induziert wird. Es setzt sich aus der Decarboxylase CadA, welche die Decarboxylierung von Lysin zu Cadaverin katalysiert, dem Lysin/Cadaverin Antiporter CadB und dem Transkriptionsregulator CadC zusammen. Bei Lysinmangel verhindert die Lysin-Permease LysP die Expression des *cadBA* Operons, während das kleine Nukleoid-assoziierte Protein H-NS die Transkription sowohl von *cadBA* als auch von *cadC* unterdrückt. Anhand einer ungerichteten Mutagenese-Studie konnte aufgedeckt werden, dass eine Deletion von *yjeK* zu einer stark reduzierten Cadaverinproduktion führt. *yjeK* kodiert für eine 2,3-Lysin-Aminomutase (LAM), welche die Isomerisation von (S)-α-Lysin zu (R)-β-Lysin katalysiert. Die verkürzte lysyl-tRNA Synthetase YjeA benutzt das entstandene (R)-β-Lysin, um den Translations-Elongationsfaktor EF-P posttranslational an einem konservierten Lysinrest (K34) zu modifizieren. Schon seit über dreißig Jahren gibt es Untersuchungen zu EF-P und seinem eukaryotischen Ortholog eIF5A, aber die genaue Funktion der beiden Proteine in Bezug auf die Translation konnte bisher nicht entschlüsselt werden.

In dieser Arbeit wurde die Rolle von modifiziertem und somit aktivem EF-P innerhalb des Cad Systems genauer untersucht. Die stark verminderte *cadBA* Expression in Δ*yjeA*, Δ$yjeK_{642\text{-}1029}$ und Δ*efp* Mutanten konnte auf eine reduzierte CadC Translation zurückgeführt werden. Da Experimente zeigen konnten, dass die Translation von *cadA* und *cadB* EF-P unabhängig ist, konnte eine allgemeine Rolle von EF-P in der Translation ausgeschlossen werden.

Die Identifikation von CadC als erstes direktes Zielobjekt von EF-P in *E. coli* erlaubte weitere Untersuchungen in Hinsicht auf die Funktion von EF-P. β-Galactosidase Tests mit CadC´-LacZ Fusionen unterschiedlicher CadC Länge in efp^- und efp^+ Zellen ergaben, dass EF-P für die Translation einer Sequenz benötigt wird, die sich in *cadC* zwischen Codon 108 und 158 befindet. Innerhalb dieser Sequenz findet man eine Gruppe von drei aufeinanderfolgenden Prolinen (Pro120-Pro121-Pro122). Der Austausch der Prolingruppe gegen Alanine führte in den Test-Zellen sogar unter nicht-induzierenden Bedingungen zu hoher *cadBA* Expression. Folglich scheint die Kopienzahl von CadC durch EF-P streng

reguliert zu werden, was für eine Stress-abhängige Regulation des Cad Systems unerlässlich ist.

Um den Wirkungsmechanismus von EF-P besser zu verstehen, wurden EF-P unabhängige CadC´-LacZ Hybride verwendet, in die artifiziell eine unterschiedliche Anzahl an Prolinen eingebaut wurde. Drei Proline reichten aus, um die Translation der Konstrukte EF-P abhängig zu machen. Dabei spielte das Prolincodon oder der Kontext der Proline im Protein keine Rolle. Außerdem war die Expression der prolinreichen Proteine AmiB, FlhC, Flk, NlpD, RzoR, TonB und UvrB ebenfalls von EF-P abhängig. Das Erkennen von drei aufeinanderfolgende Prolinen durch EF-P ist folglich ein genereller Mechanismus und nicht auf CadC begrenzt. Dr. Agata Starosta aus der Gruppe von Dr. Daniel Wilson (Gene Center, LMU München) bestätigte das Stocken der Ribosomen an polyprolinreichen Sequenzen, das durch das Fehlen von EF-P ausgelöst wird, über in vitro Translationsexperimente.

Abschließend wurde untersucht, ob die Expression und Modifikation von EF-P Stress-abhängig reguliert wird. In dieser Arbeit gibt es erste Hinweise darauf, dass die Transkription von *efp* über einen Repressor reguliert wird. Außerdem scheint die Expression von YjeA und YjeK vom pH-Wert des Mediums und der Anwesenheit des kleinen RNA-bindenden Proteins Hfq abhängig zu sein. Somit könnten auch kleine regulatorische RNAs bei der Modifikation von EF-P eine Rolle spielen.

Die Ergebnisse dieser Arbeit enthüllen einen neuen Regulationsmechanismus durch EF-P - abhängige Translation. Hunderte bis Tausende polyprolinreiche Proteine existieren in Bakterien, Archaeen und Eukaryoten. Deshalb scheinen EF-P und seine Orthologe aIF5A und eIF5A in allen drei Reichen des Lebens eine wichtige Rolle in der Anpassung der Kopienzahl von Proteinen mit unterschiedlicher Funktion zu spielen.

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Appendix

A1: Exchange of lysine residues against alanines or arginines in CadC

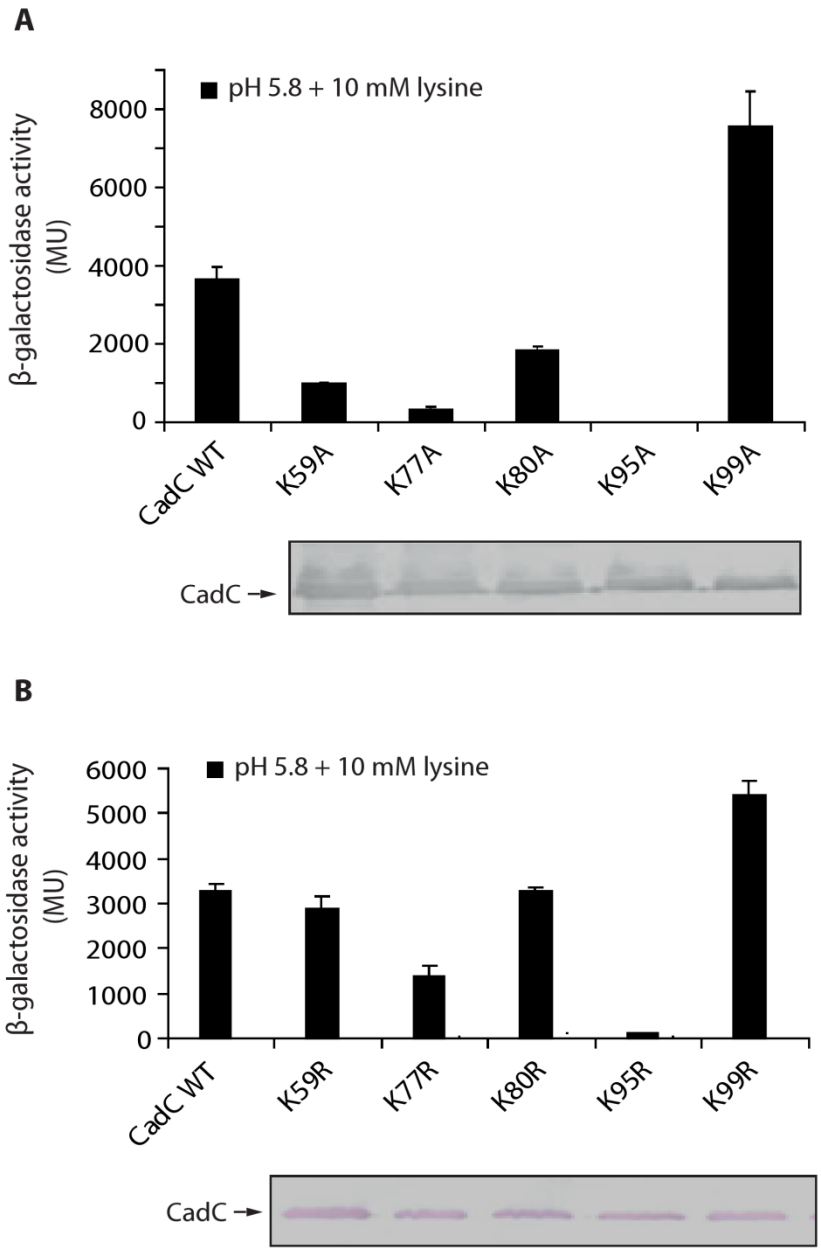

Figure A1: Exchange of lysines against alanines (A) or arginines (B) in CadC.

Single mutations were achieved by two step PCR using plasmid pET16b-CadC (Küper & Jung, 2005). Cells of strain MG-CL-123 were transformed with the resulting plasmids and were grown microaerobically in KE medium under inducing conditions (pH 5.8 with 10 mM lysine). To confirm CadC membrane integration, 10 µl of whole cells ($OD_{600} \approx 10$) (A) or 25 µg of membrane vesicles (B) were loaded onto a SDS gel. Western Blots were performed using CadC specific antibodies.

Various bacterial proteins are targets for post-translational modifications such as acetylation, pupylation, glycosylation or phosphorylation (Abu-Qarn *et al.*, 2008; Garnak & Reeves, 1979; Pearce *et al.*, 2008; van Noort *et al.*, 2012; Wang *et al.*, 2010; Yu *et al.*, 2008). These are important for protein stability and activity and often occur at lysine residues (van Noort *et al.*, 2012; Wang *et al.*, 2010; Zhao *et al.*, 2010). It was speculated, if active CadC could contain such a modified lysine residue as well. Therefore, lysine residues of the cytoplasmic domain of CadC were exchanged against alanines or arginines. Interestingly, lysine 95 led to fully abolished *cadBA* expression (A1). However, future studies have to reveal if this residue is really a candidate for post-translational modification of CadC, or if it is just very crucial for stereospecific interaction of CadC with the *cadBA* promoter DNA.

A2: β-Galactosidase activities in strain MG-CL-1 (*cadA::lacZ*) and the search for a promoter upstream of *cadA*

Fig. A2 (A) shows the β-galactosidase activities measured in strains MG-CL-1 (*cadA::lacZ*), MG-CL-1-*yjeA* and MG-CL-1-*yjeK*. The result was the same as obtained with strain MG-CR (*cadBA::lacZ*). However, β-galactosidase activities in MG-CL-1 cells grown under inducing conditions were usually twice as high as the ones measured in MG-CR cells (see Fig. 9).

To investigate, if *cadA* is regulated by a promoter additional to P_{cadBA}, several "P_{cadA}"-*lacZ* fusions were constructed. Therefore, 79 bp, 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp or 800 bp of the sequence upstream of nucleotide +1 of *cadA* were cloned into vector pBBR1-MCS3-LacZ. Cells of strain BW25113 (wildtype) were transformed with the resulting plasmids. No significant β-galactosidase activities could be measured with any of the "P_{cadA}"-*lacZ* constructs (Fig. A2, B), indicating that *cadA* is not under control of a second promoter.

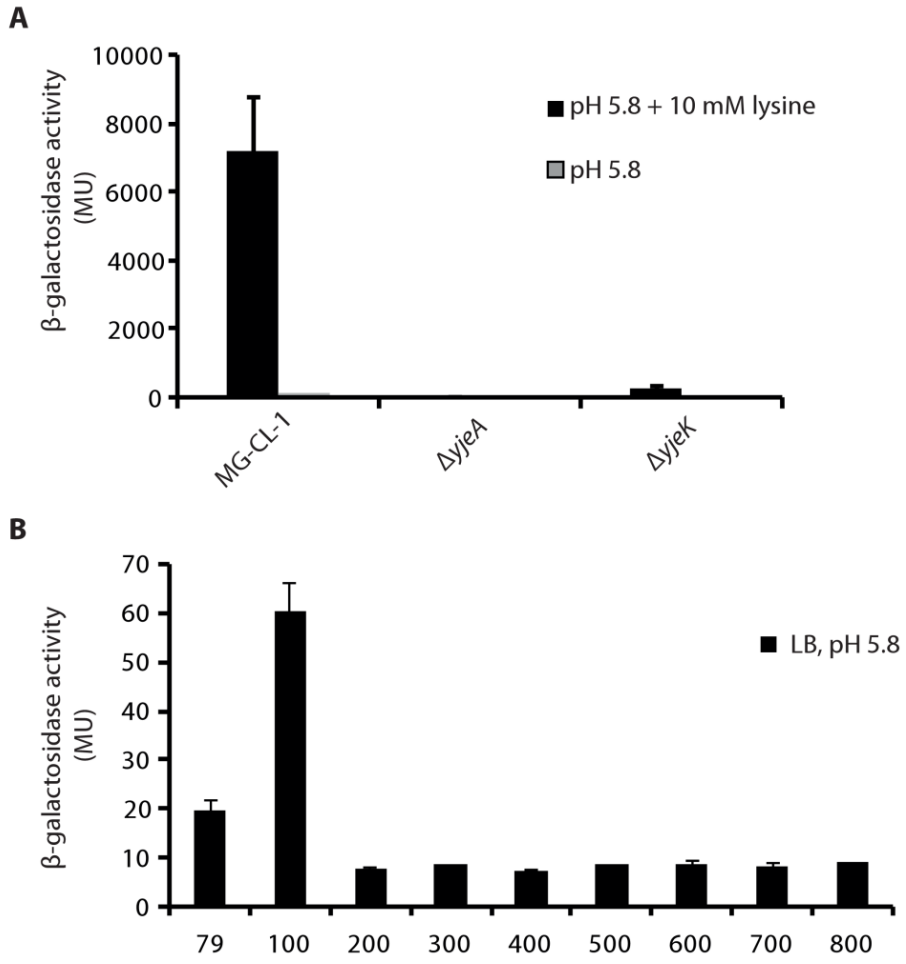

Figure A2: A) β-Galactosidase activities in cells harboring a *cadA::lacZ* mutation. Strains MG-CL-1 (MG1655 Δ*lacZ cadA*::*lacZ*), MG-CL-1-*yjeA* (Δ*yjeA*) and MG-CL-1-*yjeK* (Δ*yjeK*) were grown microaerobically in KE medium under inducing (pH 5.8 with 10 mM lysine) and non-inducing (pH 5.8) conditions.

B) "P_{cadA}"-*lacZ* fusions. Cells of BW25113 harboring various pBBR1-MCS3-P_{cadA}-LacZ plasmids were cultivated microaerobically in LB medium (pH 5.8). The length (bp) of the sequence found upstream of the transcriptional start site of *cadA* is depicted as a number (79, 100, 200 and so forth).

A3: *E. coli* proteins containing polyproline stretches

Table A1: Polyproline-rich proteins in *E. coli*.

Gene	GI	Protein
aceK	g1790446	Isocitrate dehydrogenase kinase/phosphatase
adrA	g1786584	Diguanylate cyclase for cellulose regulation
adrB	g87081980	predicted membrane-anchored cyclic-di-GMP phosphodiesterase
agp	g1787237	glucose-1-phosphatase/inositol phosphatase
amiB	g1790611	N-acetylmuramoyl-l-alanine amidase II
bcsB	g1789952	regulator of cellulose synthase, cyclic di-GMP binding
cadC	g1790576	DNA-binding transcriptional activator
clsA	g1787502	cardiolipin synthase 1
clsB	g1787007	cardiolipin synthase 2
clsC	g87081826	cardiolipin synthase 3, active in stationary phase
creC	g1790861	sensory histidine kinase in two-component regulatory system with CreB or PhoB, regulator of the CreBC regulon
cyoB	g1786634	cytochrome o ubiquinol oxidase subunit I
dppF	g1789962	dipeptide transporter
entF	g1786801	enterobactin synthase multienzyme complex component, ATP-dependent
entS	g1786806	enterobactin exporter, iron-regulated
envZ	g1789808	sensory histidine kinase in two-component regulatory system with OmpR
eutL	g1788780	predicted carboxysome structural protein with predicted role in ethanolamine utilization
flhC	g1788201	DNA-binding transcriptional dual regulator with FlhD
flk	g1788661	predicted flagella assembly protein
focB	g1788837	predicted formate transporter
ftsK	g1787117	DNA translocase at septal ring sorting daughter chromsomes
gfcC	g1787220	conserved protein
ggt	g1789856	gamma-glutamyltranspeptidase
glpR	g1789829	DNA-binding transcriptional repressor
gltB	g1789605	glutamate synthase, large subunit
gntX	g87082263	protein required for the utilization of DNA as a carbon source
gor	g1789915	glutathione oxidoreductase
gpr	g1789375	L-glyceraldehyde 3-phosphate reductase
hcaD	g1788892	phenylpropionate dioxygenase, ferredoxin reductase subunit
hisD	g1788331	bifunctional histidinal dehydrogenase/ histidinol dehydrogenase
hofM	g87082257	protein required for the utilization of DNA as a carbon source, pilus assembly protein homolog
holB	g1787341	DNA polymerase III, delta prime subunit
hyaF	g1787211	proteininvolved in nickelincorporationinto hydrogenase-1 proteins
hyfR	g87082117	DNA-binding transcriptional activator, formate sensing
insZ'	g1787491	pseudogene, transposase homolog
katE	g48994891	catalase HPII, heme d-containing
lepA	g1788922	back-translocating elongation factor EF4, GTPase
ligT	g87081692	2'-5' RNA ligase
lsrC	g1787793	autoinducer 2 import system permease protein
malG	g1790464	maltose transporter subunit

mepA	g1788668	murein DD-endopeptidase
mrcA	g87082258	fused penicillin-binding protein 1a: mureintransglycosylase/ mureintranspeptidase
mtlR	g1790029	mannitol operon repressor
nhaR	g1786202	DNA-binding transcriptional activator
nlpC	g1788001	predicted peptidase, C40 clan, lipoprotein
nlpD	g1789099	activator of AmiC murein hydrolase activity, lipoprotein
nudC	g48994995	NADH pyrophosphatase
nuoC	g1788622	NADH:ubiquinone oxidoreductase, fused CD subunit
paoB	g1786479	PaoABC aldehyde oxidoreductase, FAD-containing subunit
patA	g87082205	putrescine:2-oxoglutaric acid aminotransferase, PLP-dependent
patD	g1787715	gamma-aminobutyraldehyde dehydrogenase
pfkB	g1788017	6-phosphofructokinase II
phoB	g1786599	DNA-binding response regulator in two-component regulatory system with PhoR (or CreC)
phoR	g1786600	sensory histidine kinase in two-component regulatory system with PhoB
pka	g1788938	protein lysine acetyltransferase
ppc	g1790393	phosphoenolpyruvate carboxylase
pstA	g1790163	phosphate transporter subunit
pstS	g2367271	periplasmic phosphate binding protein, high-affinity
recB	g1789183	exonuclease V (RecBCD complex), beta subunit
recG	g2367254	ATP-dependent DNA helicase
rnb	g1787542	ribonuclease II
rsmA	g1786236	16S rRNA m(6)A1518, m(6)A1519 dimethyltransferase, SAM-dependent
rutD	g1787244	putative aminoacrylate hydrolase, reactive intermediate detoxification
rzoD	g87081757	DLP12 prophage; predicted lipoprotein
rzoR	g87081890	Racprophage; predicted lipoprotein
sdaC	g1789160	predicted serine transporter
slyX	g1789747	protein required for phi X174 lysis
tamA	g1790666	translocation and assembly module for autotransporter export, outer membrane subunit
tauC	g1786564	taurine transporter subunit
tonB	g1787505	membrane spanning protein in TonB-ExbB-ExbD transport complex
uvrB	g1786996	excinulease of nucleotide excision repair, DNA damage recognition
valS	g1790708	valyl-tRNA synthetase
yaaX	g1786186	predicted protein
yacH	g1786308	predicted protein
yaeI	g87081695	phosphodiesterase with model substrate bis-pNPP
yafZ	g87081705	CP4-6 prophage; conserved protein
yceI	g1787295	secreted protein
ycgG	g87081845	predicted membrane-anchored cyclic-di-GMP phosphodiesterase
ycgL	g1787427	conserved protein
ycgR	g1787443	flagellar velocity braking protein, c-di-GMP-regulated
ydcO	g87081909	inner membrane protein, predicted transporter, function unknown
ydeI	g1787817	conserved protein
ydiK	g1787979	inner membrane protein, UPF0118 family
yeeJ	g87082015	probable adhesin
yegI	g1788385	conserved protein
yeiG	g1788477	S-formylglutathione hydrolase

yfbK	g1788606	conserved protein
yfcO	g87082086	predicted protein
yfcR	g1788676	predicted fimbrial-like adhesin protein
yfdK	g1788696	CPS-53 (KpLE1) prophage; conserved protein
yfjQ	g1788987	CP4-57 prophage; predictedprotein
yhbW	g1789551	predicted enzyme
yhfW	g1789781	predicted mutase
yidI	g1790111	inner membrane protein
yifB	g1790201	predicted bifunctional enzyme and transcriptional regulator
yifL	g87082333	predicted lipoprotein
yjbG	g1790460	conserved protein
yjdA	g1790548	mutational suppressor of yhjH motility mutation, function unknown; related to dynamin GTPase
yodB	g87082013	cytochrome b561 homolog
ypdI	g2367134	predicted lipoprotein involved in colanic acid biosynthesis
ytfE	g1790654	iron-sulfur cluster repair protein RIC

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